

# Population and conservation genetics of the shy albatross complex



Cathryn L. Abbott

A thesis submitted for the degree of Doctor of  
Philosophy of The Australian National University

November 2004

## Declaration

The research presented in this thesis is my own original work except where due reference is given in the text. All chapters are the result of collaborative research that I carried out with others, and in Chapters I and V John Trueman contributed to phylogenetic analyses. However, I am the principal contributor to all aspects of the work, none of which has been submitted for any previous degree.

A handwritten signature in black ink, reading "Cathryn Abbott". The signature is written in a cursive style with a large, stylized 'A' and 'B'. Below the signature is a horizontal dotted line.

CATHRYN L. ABBOTT

November 2004



Dedication

*For Liza and Jenna and Sable, of course*

# Acknowledgements

The flame of conception seems to flare and go out, leaving man shaken, and at once happy and afraid... This is the greatest mystery of the human mind – the inductive leap. Everything falls into place, irrelevancies relate, dissonance becomes harmony, and nonsense wears a crown of meaning. But the clarifying leap springs from the rich soil of confusion, and the leaper is not unfamiliar with pain.

— John Steinbeck, *Sweet Thursday*

I suspect suggesting this thesis is a clarifying leap is a leap in itself, though I assure you it wasn't entirely painless. Such a great number of people nursed me through the production of this work that it's overwhelming to think of how to thank them all. I am immeasurably grateful to my supervisors Mike Double and Andrew Cockburn. Mike was dedicated, passionately interested, and incredibly helpful throughout all the stages of this research; not only did I benefit from this immensely but the quality of the work was also tremendously improved by it. I recognise and appreciate the level of commitment it took for Mike to be as accessible to me as he was. Andrew provided excellent advice and support that came in too many forms to mention, though the feedback he provided on manuscript drafts was particularly insightful. He was a constant source of clarity when I was too close to a problem to see its solution (or when I just wasn't clever enough!), and is an inspiring person to work with.

Barry Baker and Rosemary Gales provided instrumental assistance in getting this project off the ground and have given me enduring collaborative support since then. Barry helped with gaining funding, contributed valuable input on the fisheries related research, and always provided a worthy excuse for me to go somewhere new for coffee. Rosemary shared her wealth of experience in working with shy albatrosses, was very helpful with permits and access requirements, and has left me with fond memories of crunchy camp rice that make me smile about those magical days on Albatross.



It has been a pleasure and a privilege to work in as friendly and achieving a work environment as Botany and Zoology. I worked alongside great people who shared with me the joys and sorrows of doing scientific research: Rachel Alderman, Rose Andrew, Amber Beavis, Nadeena Beck, Caroline Blackmore, Chris Boland, Susan Burrows, Dave Chapple, Stu Dennis, Miranda Ebbers, Dan Ebert, Diana Fisher, Janet Gardner, Kelli Gowland, Rob Heinsohn, Anke-Maria Hoefer, Naomi Langmore, Sarah Legge, Matt Morgan, Steve Murphy, Penny Olsen, Dirk Platzen, Jess Stapley, Ian Wallis, and Bob Wong. Many of you have become dear friends. I especially thank Janet and Kelli for always being available for some decent girl talk.

Though the genetics lab is somewhat of a nemesis for many of my BoZo peers, I confess to actually liking it in there. Thanks to: Chris Hayes, for liking it too (you do so!) and sharing so many of your lab secrets with me; Ginny Sargent, for always being accessible and finding a solution to any problem; Mike Double, for recognizing that the results did not come easy and were worth getting excited about; and to everyone who kept me company.

A wealth of wild and often wonderful ideas, or a friendly hand to hold while wading down the muddled path of labwork and genetic analyses, were given to me by Theresa Burg, Lyn Cook, Mike Crisp, Rod Peakall, Anna Robinson, and Dave Rowell. In particular I thank John Trueman for his vital contribution to solving the mystery of the 'double sequence phenomenon'.

I relied heavily on many people both for help in the field and for obtaining samples from several far reaches of the southern hemisphere. They were generous in sharing their knowledge and experience with me, and patient with me while I got my feet wet in the field (quite literally at times). They are: Jacinda Amey, Nigel Brothers, Ian Flux, Anna Lashko, Gus McAllister, Pete McClelland, Peter Milburn, Janice Molloy, David Pemberton, Chris Robertson, Peter Ryan, Jenny Scott, Lindsay Smith, Dave

Stewart, Aleks Terauds, Kath Walker, and Ian West. Special thanks to Sheryl Hamilton and Alan Wiltshire for their help on all fieldwork related fronts, including floor space to crash on in Hobart. Support from the following organizations was also instrumental for sample collection in this study: New Zealand Conservation Services Levy Program, New Zealand Department of Conservation, Southern Ocean Seabird Studies Association, Southland Kaitiaki roopu, Tasmanian Museum and Art Gallery, Tasmania's Nature Conservation Branch (DPIWE).

Financial support was provided by Environment Australia, Winifred Violet Scott Estate, Gould League of NSW, Stuart Leslie (Birds Australia), and Australian Geographic. Madeleine Haag and Gary Mayo were lifesavers with respect to tackling the confusing worlds of administration and ordering lab supplies.

Thanks to Neil Smith and the *Wild Wind* for several unforgettable voyages to Albatross Island in Bass Strait. While some trips took 6 hours and another took 2 days, and anchor ropes sometimes snapped, Neil always got us there and back again. I am also hugely appreciative of 'Big Maurie' and the Tasmanian Police Marine and Rescue Division (at Stanley) for safe, reliable, fun, and FAST trips. I loved Maurie's legendary pep talk before our roughest trip: "Remember, this boat is designed to travel through the air as well as through the water; whatever you do, just don't let go!" I thank the crew of the *Marine Countess* for transporting us safely, and more importantly, smoothly, to and from the Auckland Islands in New Zealand's subantarctic. I never knew how good mutton chops for breakfast could taste!

I have family and friends near and far who deserve credit for buoying me when I needed it most. Mom, Dad, Bryna, Josh, and Kath: you are precious to me. Life in Canberra was made loads of fun by my local 'fam' in particular: Lee Halasz, Natane Halasz, and Julie Matarczyk. I miss you now that you're gone. Thanks to Allan and Meredith Gilmore for treating me like one of theirs and nurturing me with roast



dinners and decadent desserts at the farm. Finally, thanking my husband Scott feels like an unsatisfactory token for such hearty gifts; I trust that he already knows how grateful I am.

## Thesis abstract

Shy (*Thalassarche cauta*) and white-capped (*T. steadi*) albatrosses are categorised as vulnerable according to the International Union for the Conservation of Nature criteria and are incidentally killed during their interactions with fisheries. I used genetic tools to allow the first reliable separation of shy and white-capped albatrosses, and to describe their evolutionary histories, their conservation and population genetics, their unusual patterns of mitochondrial DNA evolution, the spatial distribution of fisheries bycatch mortality, and the mating system of shy albatrosses. This is the first in-depth study to investigate these species concurrently, and thereby provides extensive species-specific information that was previously unavailable for these taxa.

The first part of this thesis begins with an investigation of the evolutionary relationship between shy and white-capped albatrosses using mitochondrial DNA control region sequences (Chapter I). Phylogenetic analyses showed strong separation of a clade containing shy and white-capped albatrosses from a clade containing their closest relatives (*T. eremita* and *T. salvini*), but did not decipher species-level relationships. However, phylogeographic techniques confirmed the demographic isolation of shy from white-capped albatrosses, and suggested that shy albatrosses arose through range expansion by white-capped albatrosses. In Chapter II, I built on the results obtained in Chapter I by using polymorphic microsatellite DNA markers. I detected high levels of genetic structuring and many unshared alleles between the species, which provided strong evidence against any contemporary gene flow between them. Within each species, the three island populations of shy albatrosses were found to be genetically distinct, whereas the three island populations of white-capped albatrosses were undifferentiated. I recommend that the three white-capped albatross populations (as a whole) and each



shy albatross population be treated as separate units for conservation. All measures indicated lower genetic diversity within shy albatrosses than within white-capped albatrosses and support the hypothesis that shy albatrosses were derived from white-capped albatrosses.

In Chapter III, I used genetic data to assess the relative impact of fisheries bycatch mortality on shy and white-capped albatrosses, which had previously been precluded by difficulties identifying bycatch carcasses to species level. I describe a simple test based on a single nucleotide polymorphism in mtDNA of shy and white-capped albatrosses to determine the species composition of fisheries bycatch carcasses recovered from Australian, New Zealand, and South African waters. The accuracy of this test was found to be 98%, as evaluated using a more rigorous microsatellite-based method of provenance assignment, which was also used to determine the population of origin of bycatch shy albatrosses. Results indicated that bycatch mortality co-occurred between the two species in Tasmanian waters only; in all other zones the bycatch was exclusively comprised of white-capped albatrosses.

Genotypic provenance assignment testing correctly assigned 72% of shy albatrosses to their population of origin. This study is the first to provide insight into the relative vulnerability of shy and white-capped albatrosses to bycatch mortality across a broad spatial scale, and to establish the vast differences in their at-sea distributions.

In Chapter IV, I investigated the mating system of shy albatrosses. Data on the reproductive behaviour of procellariiform seabirds are notably lacking, and where available are inconsistent with genetic paternity data. Hence I combined genetic paternity analysis with behavioural observations during the pre-laying period to describe the copulatory behaviour of this species and to identify how males achieve within-pair and extra-pair paternity. I discovered that the vast majority of copulations occurred on the nest, were unforced, and were within-pair; and that

females controlled the success of copulations. Extra-pair paternity was detected at a low frequency (7-10%), despite male use of frequent copulation as a paternity guard.

In Chapter V, I characterized a novel mitochondrial DNA duplication near and including the control region in five *Thalassarche* albatrosses. Although it was initially thought that selection for small genome size in mtDNA would result in duplicated segments having a relatively short lifespan, recent studies have revealed ostensibly functional duplicate copies of the control region that have persisted through speciation events. Indeed, I found that the duplicate control regions aligned easily, were not identical in sequence or in length, and did not contain evidence of degeneration. Phylogenetic analyses indicated that the two control region copies were evolving largely in concert, however a short section within them was clearly evolving independently. To my knowledge this is the first time contrasting evolutionary patterns have been reported for duplicate control regions. Other evidence suggested that this duplication is present in all procellariiform seabirds, and hence needs to be considered in the development of phylogenies based on mitochondrial sequences for these taxa.



# Table of Contents

Declaration	ii
Dedication	iii
Acknowledgments	iv
Thesis Abstract	viii

General Introduction	1
----------------------	---

Chapter I: Phylogeography of shy and white-capped albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy	13
Abstract	14
Introduction	15
Methods	16
Results	25
Discussion	32
References	39

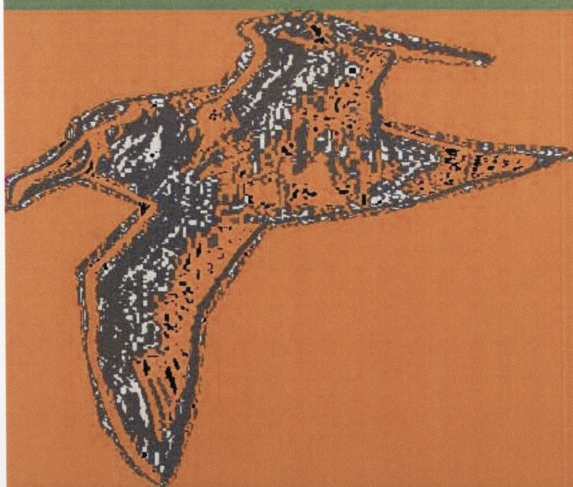
Chapter II: Genetic structure, conservation genetics, and evidence of speciation by range expansion in shy and white-capped albatrosses	47
Abstract	48
Introduction	49
Methods	52
Results	58
Discussion	63
References	69

Chapter III: Molecular provenance analysis for shy and white-capped albatrosses killed by fisheries interactions in Australia, New Zealand and South Africa	77
Abstract	78
Introduction	79

Methods	83
Results	88
Discussion	93
References	100
 Chapter IV: Copulation behaviour and paternity in shy albatrosses	 104
Abstract	105
Introduction	106
Methods	107
Results	110
Discussion	117
References	123
 Chapter V: Duplicate mitochondrial control regions in <i>Thalassarche</i> albatrosses consist of both independently and concertedly evolving segments	 126
Abstract	127
Introduction	128
Methods	129
Results and Discussion	135
References	144
 Future Directions	 149
 Appendix	 153



# General Introduction





Albatrosses spend most of their lives foraging over large tracts of ocean, favouring the rough and bountiful seas in high latitudes of the southern ocean that early sailors dubbed the 'Roaring Forties, Furious Fifties, and Shrieking Sixties'. After fledging from their natal island juveniles spend years at sea before returning to land to find a mate and breed. Reproductive rates are low; albatrosses take up to 12 years to reach breeding age (Croxall *et al.* 1990; Weimerskirch *et al.* 1987) and then produce a single chick every one or two years (Gales 1998). Mean annual adult survival rates are naturally very high, in the order of 95% (Croxall *et al.* 1990; Weimerskirch *et al.* 1987). They are amongst the longest living of birds: the oldest known albatross was over 60 years old when it ceased returning to its nest (Robertson 1993).

One of the better-known behavioural characteristics of albatrosses is their tendency to be highly philopatric, which has been confirmed in several species by demographic studies (e.g. Prince *et al.* 1994; Sagar *et al.* 1998; Weimerskirch *et al.* 1985). This has become a defining feature of the family (Diomedidae), to the extent that resulting predictions of restricted gene flow among breeding locations have led to a growing view that individual breeding islands host distinct terminal taxa (Robertson & Nunn 1998). Consistent with this, a recent taxonomic revision of albatrosses, based in part on cytochrome-*b* data (Nunn *et al.* 1996), recommended that the number of albatross genera be increased from two to four, and recognised species from 14 to 24 (Robertson & Nunn 1998). The changes to generic boundaries have not been overly contentious, however assignment of species status to many populations remains highly debated, and has prompted fine-scale molecular studies to resolve evolutionary relationships between similar species. This thesis contributes to that debate.

Burg & Croxall (2001) were the first to publish detailed species specific genetic analyses of albatrosses following the introduction of the new taxonomy (Robertson & Nunn 1998). They used mitochondrial control region sequences and microsatellite DNA markers to investigate the genetic relationships within the black-browed



albatross complex (*Thalassarche melanophris* and *T. impavida*) and within grey-headed albatrosses (*T. chrysostoma*; Burg & Croxall 2001). They followed this with a similar study on the wandering albatross complex (*Diomedea antipodensis*, *D. exulans*, *D. gibsoni*, and *D. dabbenena*; Burg & Croxall 2004). These studies provided novel and clarifying information about the taxonomy and phylogeography of these groups, and were the first to investigate population level structuring within single species. Among their taxonomic results were the discovery of a potentially cryptic species within *T. melanophris*, support for recognition of *T. impavida* as a distinct species, and support for species recognition of three of the four wandering albatross taxa, with the recommendation that *D. antipodensis* and *D. gibsoni* be reclassified as a single species (Burg & Croxall 2001, 2004). At the within-species level, Burg & Croxall (2001, 2004) found widely distributed global populations of both grey-headed albatross and the *D. exulans* type of wandering albatross to be undifferentiated and described them as globally panmictic. Similarly, a group of three geographically distant *T. melanophris* black-browed albatross populations were undifferentiated (Burg & Croxall 2001). These results not only shed light on albatross taxonomy, but they also contribute to a small but growing body of recently emerging evidence that dispersal among albatross populations can be demographically and genetically significant. More recently, Inchausti & Weimerskirch (2002) found that juvenile dispersal among *D. exulans* wandering albatross populations was sufficient to cause metapopulation structure throughout their range.

Arriving at a consensus on appropriate taxonomic boundaries in albatrosses and improving our understanding of within-species population dynamics is not only of academic value, but is particularly relevant to ongoing conservation concerns. An intimidating 83% of albatross species are categorised as Threatened under the International Union the Conservation of Nature (IUCN) criteria, which is the highest proportion of threatened species in any multi-species bird family (Croxall & Gales

1998). Concern for the future of albatross species centres around fisheries-related albatross mortality, which is occurring on a global scale and is thought to affect a large proportion of species (Croxall *et al.* 1998; Gales 1998; Inchausti & Weimerskirch 2001; Nel *et al.* 2002; Prince *et al.* 1998; Prince *et al.* 1994; Weimerskirch *et al.* 1997).

Pelagic and demersal oceanic longline fishing are both known to kill significant numbers of albatrosses (Gales 1998). This is most commonly caused when birds ingest baited hooks and drown as they are dragged under the water by the sinking line. Despite having received considerably less attention than longlining bycatch, significant albatross mortality also results from interactions with trawl fisheries (Bartle 1991, Robertson *et al.* 2003). Deaths tend to occur when birds collide with trawl warps or other cables, or become entangled in the net itself (Baker *et al.* 2002). While albatrosses have historically encountered a myriad of anthropogenic threats including introduced predators, habitat degradation, pollutants, and direct human depredation (Croxall 1998), the current threat imposed by fisheries-related mortality is potentially the most serious (Gales 1998).

Conservation efforts are typically aimed at the species level (Haig 1998), therefore an inexact taxonomy for albatrosses could lead to misguided conservation priorities. Further, even with a well-established taxonomy, taxonomic designations in themselves may not sufficiently reflect levels of genetic diversity within single species from a conservation standpoint. To guard against significant loss of genetic diversity, which corresponds to a loss in evolutionary potential, conservation priorities may need to consider individual populations or sets of populations separately (Moritz 1994). This philosophy has led to the emergence of the concept of evolutionarily significant units (ESUs; reviewed in Fraser & Bernatchez 2001), which are designed to guide conservation priorities below taxonomic levels. ESUs have been identified within many species of conservation relevance (e.g. Alpers *et al.* 2004; Hammond *et al.* 2001; Moscarella *et al.* 2003; Solorzano *et al.* 2004; Stefanni

& Thorley 2003; Waits *et al.* 2000; Zink *et al.* 2002), including black-browed and wandering albatrosses (Burg & Croxall 2001, 2004).

One of the most contentious recommendations made by the new albatross taxonomy involves two taxa, which are the main focus of this thesis. Each of four subspecies of the polytypic shy albatross (*Diomedea cauta*) was upgraded to separate species, named shy (*Thalassarche cauta*), white-capped (*T. steadi*), Salvin's (*T. salvini*), and Chatham (*T. eremita*) albatrosses (Robertson & Nunn 1998). The separation of shy and white-capped albatrosses has been hotly debated, as there is no substantial evidence for any genetic or morphological differences between them. Further, the validity of criteria used to justify the split have been questioned (Holdaway *et al.* 2001; Tennyson 2002), resulting in some recent publications recognising them as separate species (Garnett & Crowley 2000; Shirihai 2002) and others regarding them as a single species (Brooke 2004; Tickell 2000). While investigating the appropriateness of this classification is one of the aims of this thesis, for ease of discussion I use the names 'shy albatross' and 'white-capped albatross' and refer to them as species. I also use the term 'shy-type' albatrosses to refer to them collectively.

Shy albatross breeding populations are found on three islands near Tasmania, Australia, which are roughly 1800 km from the three main breeding sites of white-capped albatrosses within the Auckland Islands group in New Zealand's subantarctic. The global number of breeding pairs is estimated at 12000 for shy albatrosses and 75000 for white-capped albatrosses (Gales 1998). Shy and white-capped albatrosses are both categorised as Vulnerable under IUCN guidelines (Croxall & Gales 1998) and are thought to be suffering severe fisheries-related mortality across a wide geographic range. Estimates of longline fishing bycatch from tuna fisheries in Australian waters in 1992 indicate that shy-type albatrosses were caught in the greatest numbers, comprising 49% of the total albatross catch (Klaer & Polacheck

1997). Similarly, the abundance of shy-type albatrosses killed by tuna longliners in South African waters from 1998-2000 was higher than that of any other albatross species (Ryan *et al.* 2002). In New Zealand, more shy-type albatrosses were killed by interactions with the squid trawl fishery between 1996 and 2001 than any other seabird (Robertson *et al.* 2003).

Due to the inherent difficulties associated with observing animals that spend most of their lives at sea and breed on remote oceanic islands, genetic methods present one of the most logistically feasible means of studying them. Indeed many conservation efforts have used genetics to learn more about species that are difficult or impossible to track in the wild (e.g. Amos *et al.* 1993, Duran *et al.* 2004, Encalada *et al.* 1998, Nielsen *et al.* 1994, Page *et al.* 2004, Primmer *et al.* 1999, Watts *et al.* 2004). My aim in this thesis is to apply currently available genetic tools to gain useful and interesting information about shy and white-capped albatrosses.

My thesis is organised into five chapters. Each chapter is designed to stand alone and includes a thorough introduction to the specific topic and a review of relevant literature, therefore I have kept this general introduction brief. All chapters are written in a style suitable for publication; some have already been published and others have been submitted. It begins with two chapters in which I develop molecular markers and investigate the genetic relationship between shy and white-capped albatrosses. The following two chapters apply these molecular tools; first to assign provenance to bycatch birds, and second to examine parentage. The final chapter stems from an intriguing result I obtained while developing techniques to generate mitochondrial control region sequence data. A brief outline of my thesis is as follows.

- In Chapter I, I clarify the evolutionary relationship between shy and white-capped albatrosses through a phylogenetic and phylogeographic study using the mitochondrial control region. I also investigate the population histories of both taxa and relate them to speciation processes.
- In Chapter II, I use microsatellite DNA markers to examine the partitioning of genetic diversity within and between shy and white-capped albatrosses on a finer scale than was possible using mitochondrial DNA. I assess whether natal philopatry has led to population level genetic differentiation in these species and discuss how results impact on conservation priorities and possible ESU designations.
- In Chapter III, I develop a genetic method of distinguishing between shy and white-capped albatrosses, which overcomes our previous inability to identify shy-type bycatch carcasses to species level based on morphology. Next, I determine where and in what proportions these species are killed by fisheries-related activity in Australian, New Zealand, and South African waters.
- In Chapter IV, I use microsatellite DNA markers to determine whether shy albatrosses are always faithful to their social mates and, if not, what behavioural mechanisms lead to extra-pair young. This was prompted by results of Huyvaert *et al.* (2000) who discovered 25% extra-pair paternity in waved albatrosses, in contrast to previous evidence that colonial seabirds had low levels of extra-pair paternity. Behavioural data were not available to help interpret this surprising finding.
- In Chapter V, I investigate the source of an unexpected finding in Chapter I of apparent sequence heteroplasmy in the mitochondrial control region of shy and white-capped albatrosses and their closest relatives.

## References

- Alpers DL, Van Vuuren BJ, Arctander P, Robinson TJ (2004) Population genetics of the roan antelope (*Hippotragus equinus*) with suggestions for conservation. *Molecular Ecology*, **13**, 1771-1784.
- Amos B, Schlotterer C, Tautz D (1993) Social structure of pilot whales revealed by analytical DNA profiling. *Science*, **260**, 670-672.
- Baker GB, Gales R, Hamilton S, Wilkinson V (2002) Albatrosses and petrels in Australia: a review of their conservation and management. *Emu*, **102**, 71-97.
- Bartle JA (1991) Incidental capture of seabirds in the New Zealand subantarctic squid trawl fishery, 1990. *Bird Conservation International*, **1**, 351-359.
- Brooke M (2004) *Albatrosses and Petrels across the World*. Oxford University Press, New York.
- Burg TM, Croxall JP (2001) Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology*, **10**, 2647-2660.
- Burg TM, Croxall JP (2004) Global population structure and taxonomy of the wandering albatross species complex. *Molecular Ecology*, **13**, 2345-2355.
- Croxall JP, Rothery P, Pickering SPC, Prince PA (1990) Reproductive performance, recruitment and survival of wandering albatrosses *Diomedea exulans* at Bird Island, South Georgia. *Journal of Animal Ecology*, **59**, 775-795.
- Croxall JP, Gales R (1998) An assessment of the conservation status of albatrosses. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 46-66. Surrey Beatty & Sons, Chipping Norton.
- Croxall JP, Prince PA, Rothery P, Wood AG (1998) Population changes in albatrosses at South Georgia. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 69-83. Surrey Beatty & Sons, Chipping Norton.

- Croxall JP (1998) Research and conservation: a future for albatrosses? In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 269-290. Surrey Beatty & Sons, Chipping Norton.
- Duran S, Palacin C, Becerro MA, Turon X, Giribet G (2004) Genetic diversity and population structure of the commercially harvested sea urchin *Paracentrotus lividus* (Echinodermata, Echinoidea). *Molecular Ecology*, **13**, 3317-3328.
- Encalada SE, Bjørndal KA, Bolten AB, *et al.* (1998) Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. *Marine Biology*, **130**, 567-575.
- Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Molecular Ecology*, **10**, 2741-2752.
- Gales R (1998) Albatross populations: status and threats. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 20-45. Surrey Beatty & Sons, Chipping Norton.
- Garnett S, Crowley G (2000) *The Action Plan for Australian Birds*. Environment Australia, Canberra.
- Haig SM (1998) Molecular contributions to conservation. *Ecology*, **79**, 413-425.
- Hammond RL, Macasero W, Flores B, *et al.* (2001) Phylogenetic reanalysis of the Saudi gazelle and its implications for conservation. *Conservation Biology*, **15**, 1123-1133.
- Holdaway RN, Worthy TH, Tennyson AJD (2001) A working list of breeding bird species of the New Zealand region at first human contact. *New Zealand Journal of Zoology*, **28**, 119-187.
- Huyvaert KP, Anderson DJ, Jones TC, Duan WR, Parker PG (2000) Extra-pair paternity in waved albatrosses. *Molecular Ecology*, **9**, 1415-1419.
- Inchausti P, Weimerskirch H (2001) Risks of decline and extinction of the endangered Amsterdam albatross and the projected impact of long-line fisheries. *Biological Conservation*, **100**, 377-386.



- Inchausti P, Weimerskirch H (2002) Dispersal and metapopulation dynamics of an oceanic seabird, the wandering albatross, and its consequences for its response to long-line fisheries. *Journal of Animal Ecology*, **71**, 765-770.
- Klaer N, Polacheck T (1997) By-catch of albatrosses and other seabirds by Japanese longline fishing vessels in the Australian Fishing Zone from April 1992 to March 1995. *Emu*, **97**, 150-167.
- Moritz C (1994) Defining evolutionarily significant units for conservation. *Trends in Ecology & Evolution*, **9**, 373-375.
- Moscarella RA, Aguilera M, Escalante AA (2003) Phylogeography, population structure, and implications for conservation of white-tailed deer (*Odocoileus virginianus*) in Venezuela. *Journal of Mammalogy*, **84**, 1300-1315.
- Nel DC, Ryan PG, Crawford RJM, Cooper J, Huyser OAW (2002) Population trends of albatrosses and petrels at sub-Antarctic Marion Island. *Polar Biology*, **25**, 81-89.
- Nielsen JL, Tupper D, Thomas WK (1994) Mitochondrial DNA polymorphism in unique runs of Chinook salmon (*Oncorhynchus tshawytscha*) from the Sacramento-San Joaquin river basin. *Conservation Biology*, **8**, 882-884.
- Nunn GB, Cooper J, Jouventin P, Robertson CJR, Robertson GG (1996) Evolutionary relationships among extant albatrosses (Procellariiformes, Diomedidae) established from complete cytochrome-*b* gene sequences. *Auk*, **113**, 784-801.
- Page TJ, Sharma S, Hughes JM (2004) Deep phylogenetic structure has conservation implications for ornate rainbowfish (Melanotaeniidae: *Rhadinocentrus ornatus*) in Queensland, eastern Australia. *Marine and Freshwater Research*, **55**, 165-172.
- Primmer CR, Aho T, Piironen J, *et al.* (1999) Microsatellite analysis of hatchery stocks and natural populations of Arctic charr, *Salvelinas alpinus*, from the Nordic region: implications for conservation. *Hereditas*, **130**, 277-289.

- Prince PA, Croxall JP, Trathan PN, Wood AG (1998) The pelagic distribution of South Georgia albatrosses and their relationships with fisheries. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 137-167. Surrey Beatty & Sons, Chipping Norton.
- Prince PA, Rothery P, Croxall JP, Wood AG (1994) Population dynamics of black-browed and grey-headed albatrosses *Diomedea melanophris* and *D. chrysostoma* at Bird Island, South Georgia. *Ibis*, **136**, 50-71.
- Robertson CJR (1993) Survival and longevity of the Northern Royal Albatross *Diomedea epomophora sanfordi* at Taiaroa Head 1937-93. *Emu*, **93**, 269-276.
- Robertson CJR, Bell E, Schofield P (2003) Autopsy report for seabirds killed and returned from New Zealand fisheries, 1 October 2001 to 30 September 2002. Department of Conservation, Wellington, New Zealand.
- Robertson CJR, Nunn GB (1998) Towards a new taxonomy for albatrosses. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 13-19. Surrey Beatty & Sons, Chipping Norton.
- Ryan PG, Keith DG, Kroese M (2002) Seabird bycatch by tuna longline fisheries off southern Africa, 1998-2000. *South African Journal of Marine Science*, **24**, 103-110.
- Sagar PM, Stahl JC, Molloy J (1998) Sex determination and natal philopatry of southern Buller's mollymawks (*Diomedea bulleri bulleri*). *Notornis*, **45**, 271-278.
- Shirihai H (2002) *A complete guide to Antarctic wildlife* Alula Press, Degerby, Finland.
- Solorzano S, Baker AJ, Oyama K (2004) Conservation priorities for resplendent quetzals based on analysis of mitochondrial DNA control-region sequences. *Condor*, **106**, 449-456.
- Stefanni S, Thorley JL (2003) Mitochondrial DNA phylogeography reveals the existence of an evolutionarily significant unit of the sand goby

- Pomatoschistus minutus* in the Adriatic (Eastern Mediterranean). *Molecular Phylogenetics and Evolution*, **28**, 601-609.
- Tennyson A (2002) A recent review of New Zealand seabirds. *Australasian Seabird Bulletin*, **38**, 3-5.
- Tickell WLN (2000) *Albatrosses*. Pica Press, Sussex.
- Waits L, Taberlet P, Swenson JE, Sandegren F, Franzen R (2000) Nuclear DNA microsatellite analysis of genetic diversity and gene flow in the Scandinavian brown bear (*Ursus arctos*). *Molecular Ecology*, **9**, 421-431.
- Watts PC, Rouquette JR, Saccheri J, Kemp SJ, Thompson DJ (2004) Molecular and ecological evidence for small-scale isolation by distance in an endangered damselfly, *Coenagrion mercuriale*. *Molecular Ecology*, **13**, 2931-2945.
- Weimerskirch H, Clobert J, Jouventin P (1987) Survival in five southern albatrosses and its relationship with their life history. *Journal of Animal Ecology*, **56**, 1043-1055.
- Weimerskirch H, Brothers N, Jouventin P (1997) Population dynamics of wandering albatross *Diomedea exulans* and Amsterdam albatross *D. amsterdamensis* in the Indian Ocean and their relationships with long-line fisheries - conservation implications. *Biological Conservation*, **79**, 257-270.
- Weimerskirch H, Jouventin P, Mougins JL, Stahl JC, Van Beveren M (1985) Banding recoveries and the dispersal of seabirds breeding in French austral and Antarctic territories. *Emu*, **85**, 22-33.
- Zink RM, Drovetski SV, Rohwer S (2002) Phylogeographic patterns in the great spotted woodpecker *Dendrocopos major* across Eurasia. *Journal of Avian Biology*, **33**, 175-178.



# Chapter I



Phylogeography of shy and white-capped  
albatrosses inferred from mitochondrial  
DNA sequences: implications for  
population history and taxonomy

*Molecular Ecology* (2003) 12: 2747-2758

## Abstract

The evolutionary relationship between shy (*Thalassarche cauta*) and white-capped (*T. steadi*) albatrosses was examined using mitochondrial control region sequences. Results were interpreted in the context of a recent and contentious taxonomic revision that recommended full species status for shy and white-capped albatrosses. Low sequence divergence between shy and white-capped albatrosses (1.8%) and between their close relatives, Salvin's and Chatham albatrosses (2.9%) was observed. Much higher sequence divergence was found between the shy/white-capped pair and the Salvin's/Chatham pair (7.0%). Phylogenetic analyses confirmed the separation of the shy/white-capped pair from the Salvin's/Chatham pair but did not provide species-level resolution. Phylogeographic analyses, including a nested clade analysis,  $F_{ST}$  estimates, and an analysis of molecular variance indicated unambiguous genetic structuring between shy and white-capped albatrosses, thus confirming the demographic isolation of the species, but showed little to no structuring within each species. The geographic distribution of mtDNA haplotypes and other evidence suggest that shy albatrosses arose through range expansion by white-capped albatrosses.

## Introduction

Attempts to define appropriate taxonomic boundaries in albatrosses (Family Diomedidae) have traditionally been encumbered by a heavy reliance on incomplete specimen collections and a singular focus on morphology (Robertson & Nunn 1998). In 1998, Robertson and Nunn (1998) suggested a revised taxonomy for albatrosses based on morphology and cytochrome b sequence data presented by Nunn *et al.* (1996). It was recommended that the number of albatross genera be increased from two to four, and recognised species from 14 to 24 (Robertson & Nunn 1998). The generic changes have not been overly contentious, however a number of species status designations have remained highly debated, and have prompted molecular studies targeting the more quickly evolving mtDNA control region to resolve fine scale relationships between similar species (e.g. Burg & Croxall 2001).

The new taxonomic recommendations have been accepted as an ‘interim taxonomy’ and as the basis for further research (Alexander *et al.* 1997). Among the recommendations is the suggestion that four subspecies of the polytypic shy albatross (*Diomedea cauta*) be split into four species, named shy (*Thalassarche cauta*), white-capped (*T. steadi*), Salvin’s (*T. salvini*), and Chatham (*T. eremita*) albatrosses. The designation of shy and white-capped albatrosses as unique species has been hotly contended, as there is no substantial evidence for any genetic or morphological differences between them. Further, the validity of criteria used to justify the split have been questioned (Tennyson 2002; Holdaway *et al.* 2001), resulting in some recent publications recognising them as separate species (e.g. Garnett & Crowley 2000; Shirihai 2002) and others regarding them as a single species (e.g. Tickell 2000). I refer to them jointly as ‘shy-type’ albatrosses in this paper.

Conservation issues make an accurate understanding of the evolutionary relationship between shy and white-capped albatrosses especially important. Mortality associated

with longline fisheries is considered the biggest threat to the world's albatross populations (Croxall 1998). Like the vast majority (83%) of albatross species, shy and white-capped albatrosses are categorised as Threatened under the IUCN Red List criteria (Croxall & Gales 1998). Shy-types are reported to be suffering by-catch mortality on longline fishing hooks in Australian (Brothers 1991), New Zealand (Murray *et al.* 1993), and South African (Ryan *et al.* 2002) waters, however our inability to separate them makes it impossible to assess the impact of bycatch mortality on either one.

The aims of this study are to (1) explore the phylogenetic relationships among the four species formerly known together as *Diomedea cauta*; (2) determine the phylogeographic distribution of shy and white-capped albatross haplotypes; and (3) investigate the population histories of shy and white-capped albatrosses and the evolutionary relationship between them. I use the mitochondrial control region which is often used for intraspecies or closely-related species comparisons of avian taxa because it evolves more quickly and therefore can be more informative than cytochrome b (e.g. Avise *et al.* 2000; Burg & Croxall 2001; Crochet *et al.* 2000; Milot *et al.* 2000; Wennerberg 2001). Tree-building methods of phylogeny reconstruction,  $F_{ST}$  estimates, analysis of molecular variance, and network methods including a nested clade analysis are used.

## Methods

### Nomenclature

In accordance with the most recent taxonomic hypothesis available for albatrosses, and for ease of discussion in this paper, I have adopted the species nomenclature suggested by Robertson & Nunn (1998). I use the names 'shy albatross' and 'white-



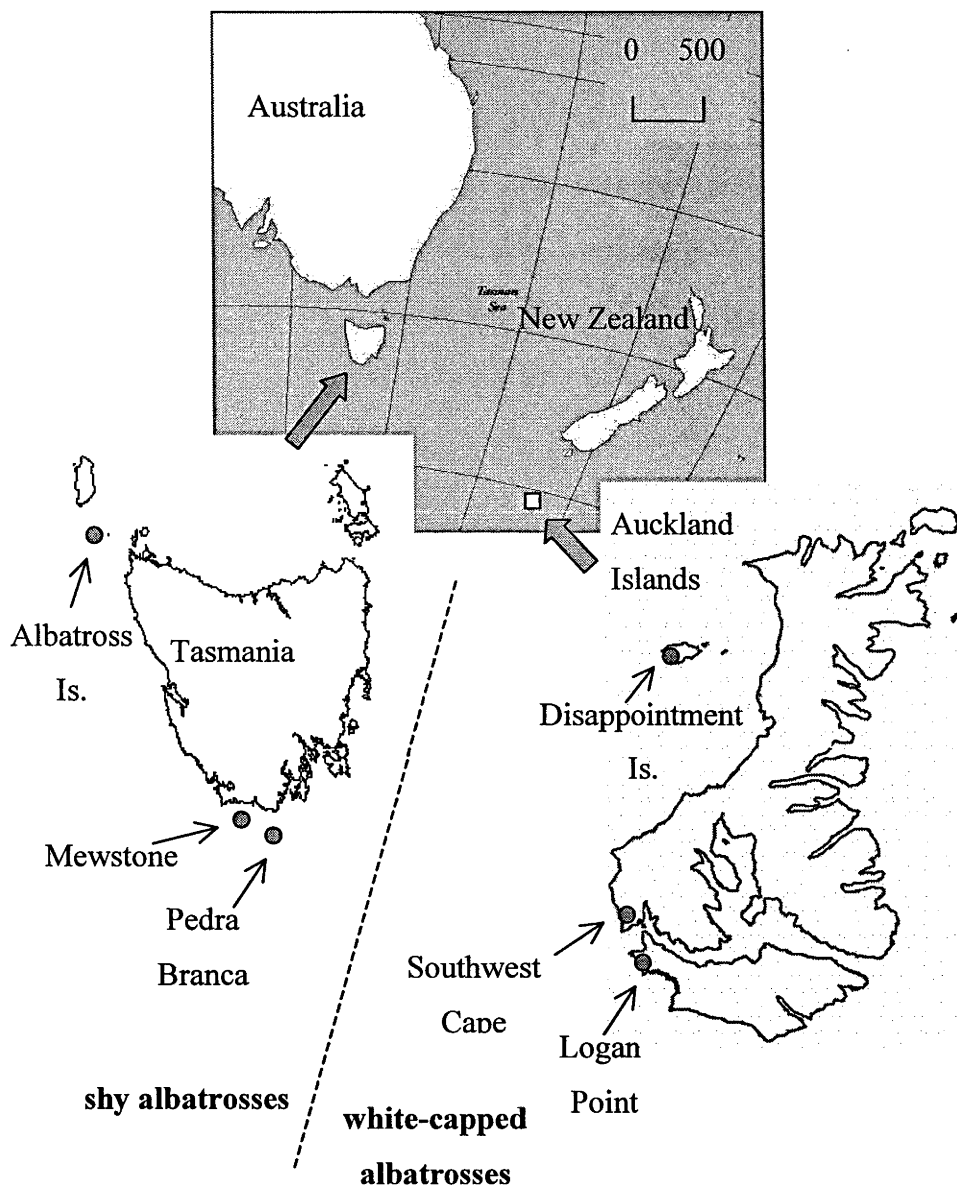
capped albatross' and refer to them as species, although my aim is to investigate the appropriateness of this classification. The term 'shy-type' is used to refer to them collectively.

### **Study species and sample collection**

Shy and white-capped albatrosses breed in separate geographic regions at slightly different times of year [shy albatrosses begin laying around the start of September whereas white-capped albatrosses begin laying in mid-November (Gales 1998)]. Shy albatrosses breed exclusively on three islands near Tasmania, Australia, which is roughly 4800 km from the main breeding sites of white-capped albatrosses in the Auckland Islands group in New Zealand's subantarctic (Figure 1). A small population of 50-100 pairs of white-capped albatrosses breed at Bollons Island in the Antipodes Islands.

I obtained DNA by taking blood from nestlings at breeding colonies. The population sizes and relative locations of breeding sites, with the number of nestlings sampled indicated in parentheses, are as follows. Shy albatross at Mewstone ( $n = 20$ ) and Pedra Branca ( $n = 20$ ) are separated by about 50 km and have *c.* 7000 and *c.* 250 breeding pairs respectively (Brothers *et al.* 1998). Approximately 420 km away is a shy albatross population on Albatross Island ( $n = 20$ ) with *c.* 5000 breeding pairs (Brothers *et al.* 1998). The three white-capped albatross breeding sites in the Auckland Islands are located within approximately 30 km of each other (population sizes taken from Gales 1998): *c.* 72000 pairs on Disappointment Island ( $n = 20$ ); *c.* 3000 pairs at Southwest Cape, Auckland Island ( $n = 19$ ); and *c.* 100 pairs at Logan Point, Adams Island ( $n = 20$ ). Samples from the small population of white-capped albatrosses at Bollons Island were not obtained. Both species lay only one egg per year, hence sampling siblings was not a concern, though I also purposely sampled chicks that were distributed as evenly throughout the colony as possible to reduce the





**Figure 1.** Locations of sampled breeding sites for shy and white-capped albatrosses. Sample sizes per population are as follows: Albatross Island ( $n = 20$ ), Mewstone ( $n = 20$ ), Pedra Branca ( $n = 20$ ), Disappointment Island ( $n = 20$ ), Southwest Cape ( $n = 19$ ), and Logan Point ( $n = 20$ ).

probability of sampling related individuals at adjacent nests. A minimum of five nests separated the nests of sampled chicks, except at Logan Point where small population size demanded more intensive sampling.

Tissue samples from three Salvin's albatrosses, three Chatham albatrosses, and two Buller's albatrosses (*Thalassarche bulleri*) were obtained from specimens from the New Zealand Conservation Services Levy Program and provided to us by the New Zealand Department of Conservation and CJR Robertson. Both blood and tissue samples were stored in 70% ethanol.

### **DNA extraction and sequencing**

DNA was extracted using ammonium acetate as described in Nicholls *et al.* (2000).

As there were no control region primers published for albatrosses at the time, I obtained sequence from cytochrome b and 12S genes for shy albatrosses using universal primers (Kocher *et al.* 1989; Simon *et al.* 1994). Within these regions I designed specific primers (forward primer cbF1: 5'-

ATGAATCGGCAGCCAACCAGTAG-3' and reverse primer 12sR1: 5'-

TAAGCGTTTGTGCTCGTAGTTCTC-3') that amplified a 3000 bp fragment of mtDNA containing the control region. I obtained complete sequence for this region by repeatedly sequencing, designing internal primers, and sequencing again until I had 'walked-through' the entire fragment.

The initial 'long-range' PCR using cbF1 and 12sR1 was done using the Expand High Fidelity PCR System (Boehringer Mannheim). Each reaction contained 100-200 ng of genomic DNA, 200  $\mu$ M dNTP, 10 pmol of each primer, and 2.6 U of Expand High Fidelity enzyme mix in 1x reaction buffer in a 50  $\mu$ l reaction volume. The cycling parameters were: 94 °C for 2 min; 30 cycles of 5 s at 94 °C, 30 s at 65 °C, 2.5 min at 72 °C and one final cycle for 7 min at 72 °C. Following amplification PCR

products were run in a 2% agarose gel, and then purified using the Bresaclean DNA Purification Kit (Bresatec). I sequenced 2-5 µl of purified PCR product using Applied Biosystems BigDye sequencing system and 1.6 pmol of primer in a 10 µl reaction volume. Products were then run on an ABI Prism 377 automated sequencer. Internal sequencing using nested primers followed the same protocol.

Domain I was the only readily amplifiable, variable segment of the control region: long repeats in Domain III made sequencing and subsequent alignment problematic. Similar complex repeats have been reported in mtDNA of other albatross (Burg 2000) and seabird (Berg *et al.* 1995) species. Domain II was highly conserved between shy-types and therefore uninformative at the level I was targeting. Forward primer GluF6 (5'-AGGATTAGACGCAACTGCCAGC-3') and reverse primer GluR6 (5'-GACGCCCCCAAAAAAAAAAAGG-3') were designed for specific amplification of 600 bp containing all of Domain I and flanking regions using the same PCR reaction conditions and cycling parameters stated above except that an annealing temperature of 55 °C was used. PCR products were gel purified using Concert Rapid Gel Extraction System (Life Technologies). Approximately 340 bp of sequence were obtained using an internal primer GluR7 (5'-CGGGTTGCTGATTTCTCG-3') because it yielded superior sequence quality compared to GluR6.

Initial amplifications yielded ambiguous sequence due to the consistent occurrence of double peaks at some sites. To investigate the source of the ambiguity, PCR products were cloned into pGEM-5Zf(+) according to the protocol supplied by the manufacturer (Promega), and transformed using heat shock into JM109 Competent Cells (Promega). Several clones were sequenced for one individual of each species. Two unique sequences were identified within single individuals for all species. New forward primers were designed that specifically amplified each copy with GluR7. SPECF1 (5'-CAGCTTATGTATAAATGCG-3') amplified a copy referred to as F1,

and SPEC2 (5'-AACAGCCTATGTGTTGATGT-3') amplified a copy referred to as F2. All F1 copies were found to contain a recombination signal on analysis using the reverse successive weighting method of Trueman (1998). Mitochondria are not known to recombine, hence F1 copies were thought to be nuclear pseudogenes, which are commonly reported in birds (Allende *et al.* 2001; Kidd & Friesen 1998; Ruokonen *et al.* 2000; Sorenson & Fleischer 1996). Several recombinant mitochondrial pseudogenes have been reported in *Conirostrum* species by Nielsen and Arctander (2001). As there was no evidence of recombination in any of the F2 copies, they were presumed to be of mitochondrial origin and targeted in this study.

### **Molecular data set**

My molecular data set comprised a 299 bp fragment of Domain I of the mitochondrial control region for each of 30 shy, 29 white-capped, three Salvin's, three Chatham, and two Buller's albatrosses. Sample sizes used in the analyses varied with the nature of the technique, as described in relevant sections below. Sequence quality and base-calling accuracy were evaluated by viewing chromatograms using Sequencer 3.0 (Gene Codes Corporation). All sequences aligned easily using ClustalX version 1.8 (Thompson *et al.* 1994).

### **SNP Test**

Initial control region sequencing of 59 birds revealed one substitutional change that consistently separated shy and white-capped albatrosses (see Figure 2). A simple single nucleotide polymorphism (SNP) test was developed using this site following the approach outlined in Fitzsimmons *et al.* (1997) as a time- and cost-effective means of screening a larger sample size to test the consistency of this pattern. Ten more birds from each of the six main shy-type breeding islands, all of which were blood-sampled as chicks at the nest, were screened.

Variable sites	
	1111111111111111111111111111222222222222222222222
	24445666777889990000001122223344455567799000001112222333344999
	44676678456076780123455601231537845682369134573790167467838124
s1 (5)	TGGTCGAGCGTACATGCAGGGCGTGAAGTAAGTGGTAATTAGGAGGCTTGCTCGAGTGAATT
sS1	.....A.....G.....
sS4	.....G.....
sS7	.....A.....G.....A.....A.....
sS10	..T.....G.....A...T.C.....A.....
sS5	.....C...G...G.....A.....
sS9	.....AT.....
sS2	..T.....G.....A...T.....A.....
sS11	.....G.....A.....
sS14	.A.....G...N.....
sL10	.....G.....A.....
sL2 (2)	.....A.....
sL3 (2)	...N.....G.....A.....
sL21	.....G.....C...A...G...A.....
sL4 (2)	.....A.....
sL25	.....T...AA.....C...A.T.....
sD38	..C.....A.....
sD50	.....C.....
sD16	..T..T.....G.....AA.....
sD5	...T.....G.....G..G..A.....
sD41	.....G.....
sD7	.....A.....A.....
cA11	.....G.A.....G.....A.....G...A.....
cA26	.....AC.G.....G.....
cA32	.....G.....N.A...C
cA65	C.....AC.G.A.....G.....
cA85	.....A...G.....A.....A.....
cA1 (2)	.....C.G.....G.....A.....
cM30	.....G.....A.....A.....
cM38	.....G.A..AC...G.....A.....A.....
cM40	.....G...A.....
cM45	.....G.....T.....
cM2 (3)	.....G.....
cP33	...T.....G.....A.....
cP3 (2)	.....G.....G.A.....G.....A.....A.....
cP4 (2)	.....A.....G.....A.....AG...
c5 (11)	.....G.....G.....A.....
Sal10	....AG....GG..GT...ACAG...G.G.....AA.....AT..A..C..G..
Sal15	.....G.....G..GT...ACAG..A...G.A..G...AA.....AT..A..C..G..
Sal17	C.....G.....GT...T.C.G.....GA.C.....AA.....AT..A..C..G..
Ere1	.....GT...ACAG..A...AG.....AA.A...AT..A..C..G..
Ere2 (2)	.....G...GT...ACAG...AG.....AA.A...AT..A..C..G..
Bul5	.TT..T..T.CG.G.ATG...ACA.G...GG.A...CG.AA.A...A.CT.G.C....
Bul9	.TTC.T..TATG.G.AT....AC.G...GA.A...CG.AA.AA.C...CT.GACA....

**Figure 2.** Variable sites in the mitochondrial control region (Domain I) of all shy (c), white-capped (s), Salvin's (Sal), Chatham (Ere), and Buller's albatrosses (Bul) sequenced in this study. The fixed difference between shy and white-capped albatrosses is highlighted. Haplotypes shared by multiple individuals have the sample size in parentheses. Most haplotype codes for shy and white-capped albatrosses include a capitalised letter depicting sampling location as follows: S = Southwest Cape, L = Logan Point, D = Disappointment Island, A = Albatross Island, M = Mewstone, and P = Pedra Branca. Haplotype s1 was found in individuals from Disappointment Island ( $n = 4$ ) and Logan Point ( $n = 1$ ). Haplotype c5 was found in individuals from Pedra Branca ( $n = 5$ ), Mewstone ( $n = 3$ ), and Albatross Island ( $n = 3$ ). Sequence identity to s1 is indicated by a '.'.

PCR amplifications for the SNP test generated a 183 bp fragment using the mismatched forward primer (TEST F4t 5'-CACTTAAACGGATTAAACCCATGAYT-3') and GluR7. Reactions consisted of 50 ng of genomic DNA, 200  $\mu$ M dNTP, 3.2 mM MgCl<sub>2</sub>, 2 pmol of each primer, 0.5 U of AmpliTaq DNA Polymerase (Applied Biosystems) in 1x reaction buffer in a 10  $\mu$ l reaction volume. PCR cycles were 2 min at 94 °C; a 'touchdown' of 25 s at 94 °C, 25 s at 65 °C to 55 °C (dropping 5 °C per 2 cycles), 25 s at 72 °C; 30 cycles of 25 s at 94 °C, 25 s at 50 °C, 25 s at 72 °C and one final cycle of 3 min at 72 °C. PCR products were digested with 3 U of *Hinf*I (Pharmacia Biotech) at 37 °C for at least one hour in a 15  $\mu$ l reaction volume. Digestion products were run in a 3% agarose gel to resolve the 26 bp difference between cut products produced by shy albatross samples and uncut products produced by white-capped albatross samples.

### Analysis

Phylogenetic analyses were performed using sequence data from 12 shy, 12 white-capped, three Salvin's, three Chatham, and two Buller's albatrosses (as outgroup). Trees were constructed in PAUP\*4.0b10 (Swofford 2002) using both unweighted cladistic parsimony and Maximum Likelihood (ML) methods. Evolutionary process models for the ML analyses were optimised in Modeltest 3.06 (Posada & Crandall 1998). The robustness of the tree estimates was assessed by bootstrapping (Felsenstein 1985) as implemented in PAUP.

Phylogeographic analyses used sequence data from ten shy albatrosses from each of the three breeding islands, ten white-capped albatrosses from Disappointment Island and Adams Island, and nine white-capped albatrosses from Auckland Island. Arlequin Version 2.001 (Schneider *et al.* 2000) was used for the analyses and descriptive statistics described here. Total molecular variance within shy and white-capped albatrosses was partitioned into hierarchical components using separate

analyses of molecular variance (AMOVA; Excoffier *et al.* 1992). Pairwise  $F_{ST}$  estimates were generated to investigate genetic differences within and between shy and white-capped albatross populations, whereby probability values for  $F_{ST}$  derive from null distributions generated from 1023 random permutations among populations. As correcting for multiple hits was unnecessary due to low divergence, uncorrected sequence divergence estimates were calculated from Nei's average number of pairwise differences within and between populations (PiXY; Nei & Li 1979).

A nested clade analysis (NCA) was also performed to examine the population structure and population histories of shy and white-capped albatrosses. NCA is aimed at "separating population structure from population history as sources of geographical associations" among haplotypes (Templeton *et al.* 1995:768), and has recently become a popular tool for analysing intraspecific genetic data (e.g. Gomez-Zurita *et al.* 2000; Paulo *et al.* 2002; Perkins 2001; Worheide *et al.* 2002). A statistical parsimony network using a 95% confidence limit was estimated for shy and white-capped albatross haplotypes and subsequently converted into a nested cladogram using the program TCS version 1.13 (Clement *et al.* 2000) and following nesting rules outlined in Crandall (1996), Templeton *et al.* (1987), and Templeton & Sing (1993). The program GeoDis 2.0 (Posada *et al.* 2000) was used to test for significant associations between haplotypes and geography using: 1) a nested contingency analysis, which treats sampled locations as categorical variables; and 2) a nested geographical distance analysis, which incorporates information of geographical distances between sampled locations. The latter analysis calculates two main statistics: the clade distance ( $D_c$ ) which measures the geographical spread of a clade, and the nested clade distance ( $D_n$ ) which measures the geographical spread of a clade in relation to other clades nested in the same higher-level category (Posada *et al.* 2000). An inference key first presented in the appendix of Templeton *et al.* (1995) simplifies interpretation of the distance statistics when the null hypothesis of

no geographical association among haplotypes is rejected. I used an updated version of the inference key to interpret the population structure and history of all clades that yielded significant results ( $P < 0.05$ ) in the nested geographical distance analysis. In-depth details on nested clade analyses are described in Templeton (1998).

## Results

Forty-three variable sites were found among the 59 shy and white-capped albatross sequences, which defined 37 unique haplotypes: 15 within shy albatrosses and 22 within white-capped albatrosses. Among the 66 total sequences generated from shy, white-capped, Salvin's, Chatham, and Buller's albatrosses, there were 44 unique haplotypes defined by 62 variable sites (Figure 2). No haplotypes were shared between shy and white-capped albatrosses and one fixed nucleotide difference was identified between the two species. Average pairwise sequence divergence between shy and white-capped albatrosses (1.8%) was less than that between Salvin's and Chatham albatrosses (2.9%). Sequence divergence between these two groups was much higher (7.0%).

## Phylogenetics

Fifty-six variable sites were found among the 32 sequences used in the phylogenetic analyses on shy, white-capped, Salvin's, Chatham, and Buller's albatrosses; 42 of which were parsimony-informative. Cladistic parsimony produced 12 trees in all of which a Salvin's/Chatham group is sister to a shy/white-capped group. There was no clear resolution of the branching order within either of these groups, and bootstrap support for the Salvin's/Chatham group was low (51%), implying that its monophyly is not supported by the data.



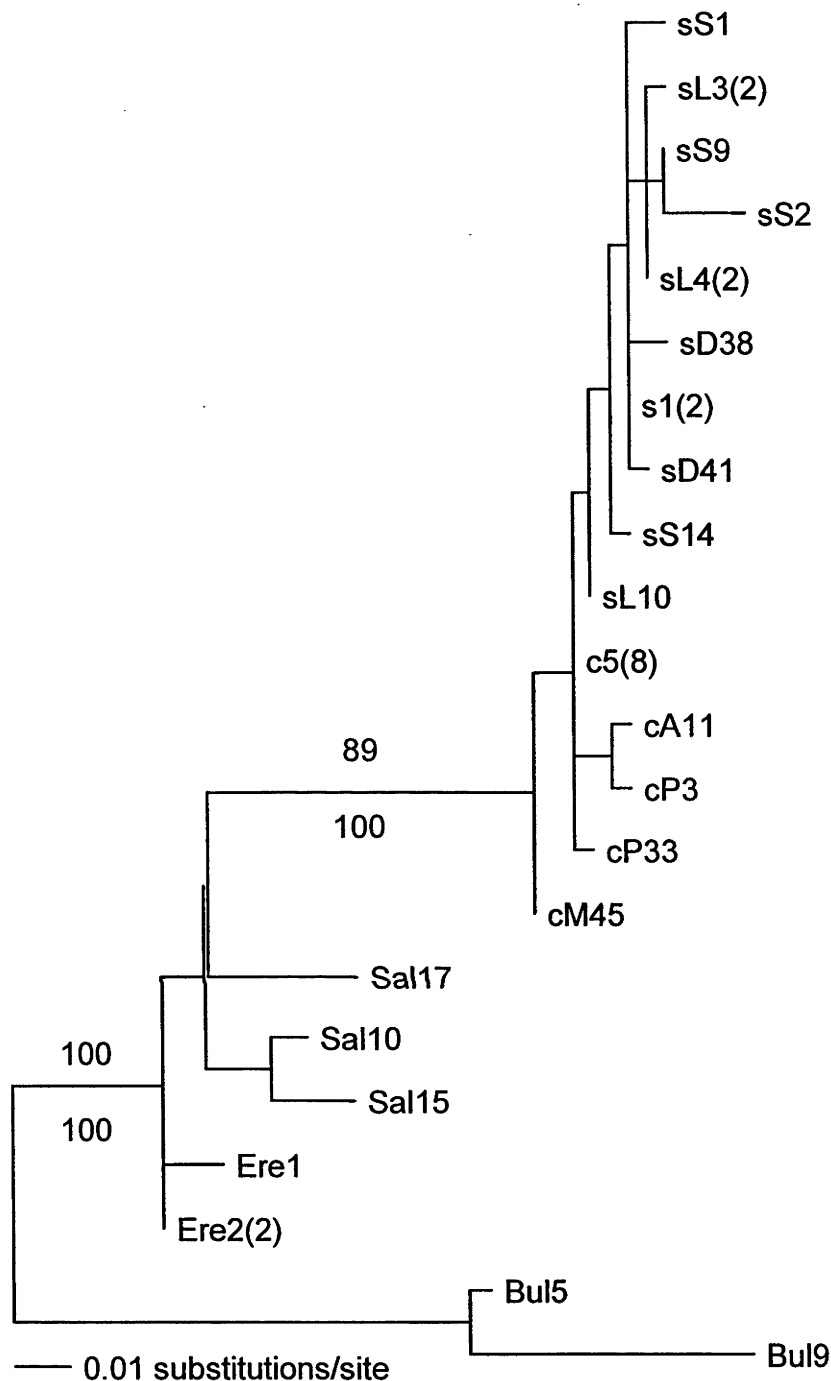
Modeltest (Posada & Crandall 1998) applied to the whole of the data suggested a highly unusual maximum likelihood model in which base-transitions and G-T transversions are common but all other transversions are disallowed. On inspection of the aligned data, three variable sites (nos. 46, 102 and 148) were seen to carry apparent G-T transversions that, because of their distribution across the taxa, raised serious doubts concerning: (1) the homology of identical states; or (2) the independence of the apparent transversion event from transitions in immediately adjacent positions. These three characters were excluded from the analysis.

Modeltest applied to the remaining characters recovered a maximum likelihood model in which parameter values were biologically reasonable. Application of this model gave the ML tree in Figure 3, shown with parsimony and ML bootstrap results.

Results from maximum likelihood and parsimony both suggest a monophyletic but internally unresolved shy/white-capped group which is well separated from an unresolved Salvin's/Chatham group. The latter is paraphyletic or else not convincingly monophyletic. Further testing of branches using models optimised with a variety of combinations of the 'suspect' and adjacent positional characters found no significant variation from these bootstrap scores (data not shown), further confirming these results.

### **Population genetics**

The various population genetic analyses of 30 shy and 29 white-capped albatrosses yielded consistent results. AMOVA showed a small yet statistically significant proportion of the variance residing among populations within each of the two species (2.6%,  $P < 0.05$ ), and larger, highly-significant components of variance existing between the shy and white-capped albatross species (37.9%,  $P < 0.001$ ) and within populations (59.4%,  $P < 0.001$ ). Pairwise  $F_{ST}$  values between populations ranged



**Figure 3.** Maximum likelihood (ML) phylogenetic tree (using TrN + I model) for the mitochondrial control region of shy (c), white-capped (s), Salvin's (Sal), and Chatham's (Ere) albatrosses, with Buller's (Bul) albatrosses as the outgroup. Three data characters were excluded from the analysis (see text). Bootstrap values over 80% obtained from 100 resamplings using ML (above branches) and parsimony (below branches) are shown. Sequences found in more than one individual have the sample size in parentheses (but note that not all sampled individuals were included in this analysis).

from -0.01 (interpreted as zero) between Logan Point and Disappointment Island, to 0.50 between Pedra Branca and Disappointment Island (Table 1).  $F_{ST}$  values for all excepting one (Mewstone/Pedra Branca,  $P < 0.05$ ) within-species population level comparisons were non-significant, and all between-species population level comparisons were highly significant ( $P < 0.001$ ).

### **Phylogeography and nested clade analysis**

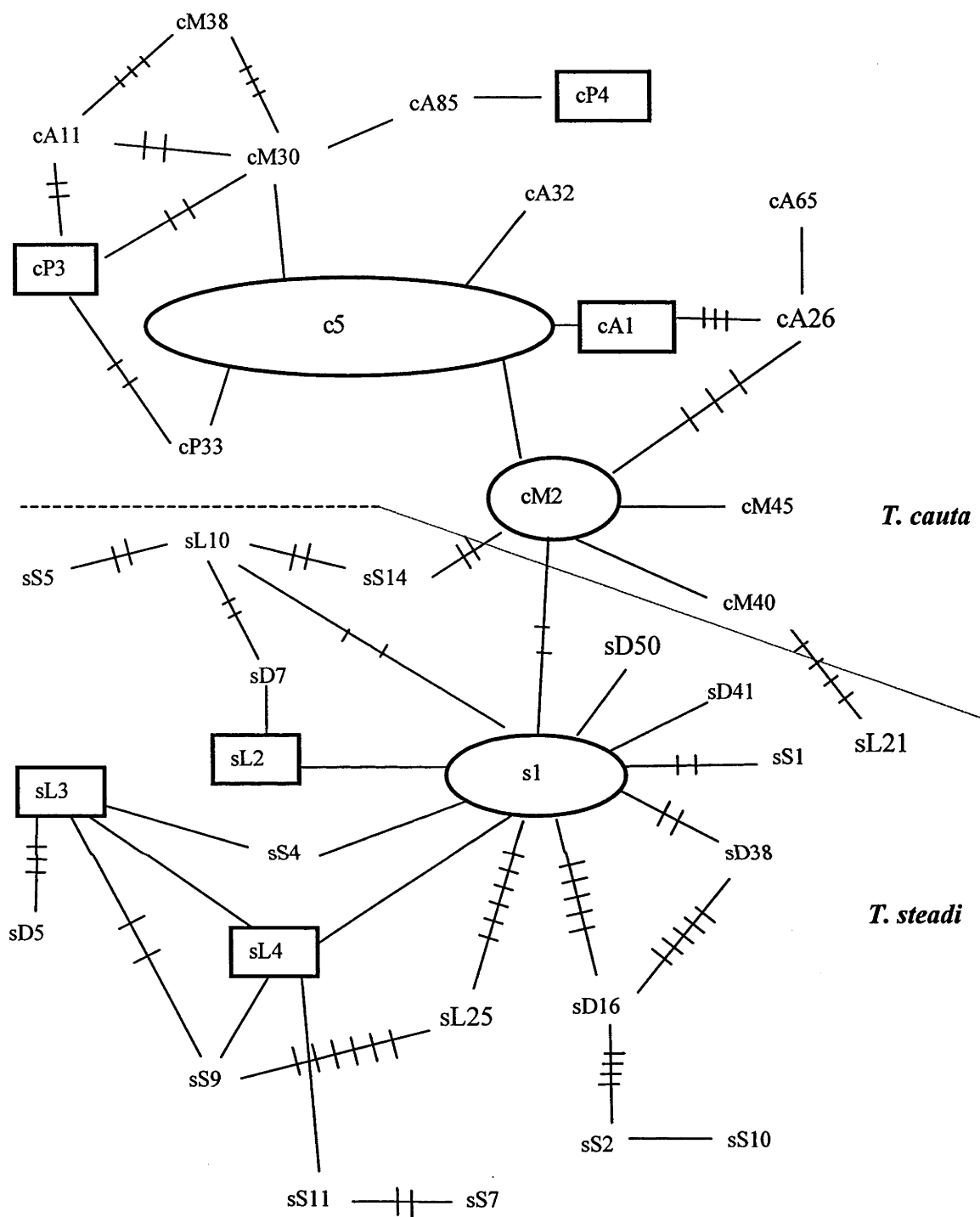
The minimum spanning network (MSN; Figure 4) depicts shy and white-capped albatrosses as two distinct groups connected by as little as one fixed substitutional change, with one white-capped albatross haplotype as an exception (sL21). This outlier is four mutational steps from the nearest shy albatross haplotype (cM40), five steps from one other shy haplotype (cM2), and is over five steps from other shy haplotypes. However it is five mutational steps from four white capped albatross haplotypes. Its correct association appears ambiguous therefore it was excluded from the NCA.

During the nesting procedure of the NCA, loops in the statistical parsimony network lead to ambiguities in the nesting design. In these cases I placed geographically more proximate haplotypes or clades together to prevent significant results arising artificially from the nesting procedure itself.

The whole network was included in four three-step clades (Figure 5). The geographical distribution of clade 3.2 corresponded to the shy albatross breeding range, that of both clades 3.1 and 3.4 corresponded to the white-capped albatross breeding range that was represented in this study, and the distribution of clade 3.4 covered Disappointment Island and Southwest Cape only. The null hypothesis of no geographical association of clades was rejected by the nested contingency analysis in two two-step clades, 2.1 ( $\chi^2 = 14.0$ ;  $P < 0.05$ ) and 2.4 ( $\chi^2 = 18.2$ ;  $P < 0.01$ ), and one

**Table 1.** Matrix of pairwise comparisons of  $F_{ST}$  (above diagonal) and their probability values (below diagonal) for three populations of shy albatrosses and three populations of white-capped albatrosses (W.C.). Values significant at  $P < 0.05$  are marked with an asterisk.

Species	Population	Shy albatrosses			White-capped albatrosses		
		Albatross	Mewstone	Pedra	Auckland	Adams	Disappt.
Shy	Albatross	—	0.07	0.07	0.34*	0.41*	0.44*
	Mewtone	0.12	—	0.16*	0.33*	0.38*	0.42*
	Pedra	0.11	0.03	—	0.37*	0.45*	0.50*
W.C.	Auckland	< 0.001	< 0.001	< 0.001	—	0.02	0.03
	Adams	< 0.001	< 0.001	< 0.001	0.28	—	-0.01
	Disappt.	< 0.001	< 0.001	< 0.001	0.20	0.63	—



**Figure 4.** Minimum spanning network from 299 bp of control region sequence from 30 shy albatrosses (c) and 29 white-capped albatrosses (s). The size of each oval is approximately proportional to the number of birds sharing that haplotype; the rectangles represent haplotypes shared by two individuals (see Figure 2 for sample size and sampling location(s) of each haplotype). The number of substitutional changes between haplotypes is indicated by the number of crosshatches. Lines with no crosshatches indicate a single base change.





three-step clade, 3.1 ( $\chi^2 = 10.8$ ;  $P < 0.05$ ). Clade 2.1 did not have correspondingly significant results in the geographic distance analysis, however clades 2.4 and 3.1, that were each comprised of four nested lower-level clades, each had one clade with significantly small and/or large Dc/Dn values. In both cases, the geographical pattern identified by the inference key was restricted gene flow with isolation by distance.

The entire cladogram showed highly significant ( $\chi^2 = 67.4$ ;  $P < 0.001$ ) associations between geography and sequence types. The inference key predicted that range expansion through long distance colonisation from the white-capped albatross range to the shy albatross range. Each of the three-step clades nested within the entire cladogram had significantly small Dc values, and clade 3.2 (which was comprised exclusively of shy albatrosses, who were absent from all other three-step clades) also had a significantly large Dn value. The coupling of a small Dc and large Dn for clade 3.2 is suggestive of long-distance movements.

### **SNP Test**

All of the 60 known-provenance birds that were subjected to the SNP test were correctly identified, thus implying that the substitutional change is indeed a fixed difference between shy and white-capped albatrosses.

### **Discussion**

I found low levels of sequence divergence in the mitochondrial control region between shy and white-capped albatrosses. Phylogenetic analyses aimed at reducing the current ambiguity around their taxonomic status did not resolve species-level relationships. However novel insight into the degree of genetic disparity between

shy and white-capped albatrosses and the evolutionary relationship between them was gained using analytical tools designed for intra-specific comparisons. Similar findings of low control region divergence have been reported in other closely related albatross species (Burg 2000).

Shy and white-capped albatrosses do not form phylogenetically distinct groups, though there is a fixed difference between them. The absence of shared haplotypes is unlikely to be an artifact of high haplotype diversity and inadequate sample sizes because the AMOVA analysis shows that the between-species component of molecular variance is 15 times higher than the within-species component. Hence it can be inferred that the lack of haplotype sharing indicates that females do not migrate between the geographic areas in which the forms occur. The low divergence observed between shy and white-capped albatrosses suggests that this demographic isolation is recent, as does the starburst pattern in Figure 4.

### **Phylogeny**

Phylogenetic analyses showed that the shy and white-capped albatross group is monophyletic, but the Salvin's and Chatham albatross group is not monophyletic. These analyses did not provide resolution to species level within either group. This is surprising with respect to the Salvin's and Chatham albatross group considering the gross morphological differences between them. Adult Chatham albatrosses have very dark plumage on the head and neck and unmistakably bright yellow bills, while Salvin's albatrosses have noticeably lighter grey head and neck plumage and grey bills with a contrasting pale culminicorn (Tickell 2000). Conversely, it is less surprising that phylogenetic methods did not resolve the shy and white-capped albatross pair given their morphological similarity.

### Low mtDNA divergence

The control region is comprised of three subregions that exhibit strikingly different levels of sequence variation (Baker & Marshall 1997) hence it is only appropriate to compare divergence estimates among studies that examine the same subregion(s). I am aware of only a few studies describing variation in Domain I of the control region between closely-related avian taxa. Sequence divergence in this subregion between two subspecies of sage grouse (*Centrocercus urophasianus*) averaged 17.5%, and contributed to the recommendation that they be recognised as separate species (Oyler-McCance *et al.* 1999). In contrast, sequence divergence in Domain I of the control region among four separate species of gnatcatchers (*Poliioptila* spp.) averaged 3.8% (Zink & Blackwell 1998). Given that shy and white-capped albatrosses are provisionally considered separate species, based on these comparisons the level of sequence divergence between them (1.8%) is low.

Low levels of genetic divergence have also been found in Domain I of the mitochondrial control region of other albatrosses. Very low divergence (about 1%) in this region has been reported between two New Zealand endemic species of wandering albatrosses that differ in breeding plumage colouration (*Diomedea gibsoni* and *D. antipodensis*; Burg 2000). In contrast, global black-browed albatross (*T. melanophrys*) populations form two distinct groups (4% sequence divergence in Domain I of the control region; Burg & Croxall 2001) that do not differ phenotypically. Slow rates of mitochondrial DNA evolution have been reported in albatrosses (Nunn & Stanley 1998), however inconsistencies in the relationship between mitochondrial control region divergence levels and morphological change in albatrosses require further investigation. Perhaps low control region divergence between easily recognisable species of albatrosses is simply attributable to a slow rate of molecular evolution in those taxa; thus divergence levels do not reflect morphological change to the degree we predict. Alternatively, the rate of molecular change in the control region may be highly uniform among albatross species, and

rapid radiation events are generating accelerated rates of morphological change between certain closely related groups. Regardless of the mechanism, the control region sequence did not substantiate all of the species boundaries suggested by Robertson and Nunn (1998).

### **Shy and white-capped albatrosses**

The minimum spanning network, AMOVA, and highly significant  $F_{ST}$  estimates show distinct structuring between shy and white-capped albatrosses and little or no structuring within each species. Together with a fixed substitutional change this is strong evidence against the occurrence of any contemporary female-mediated gene flow between these species. The SNP test developed to confirm the fixed difference could now be used as a simple, cost-effective method of identifying the species of shy-type birds killed on longline hooks.

The general lack of genetic structuring within each species may indicate that conspecific populations are exchanging individuals to a sufficient extent to prevent population level genetic differentiation. Alternatively the populations within each species could have been recently isolated, or products of recent colonisation events so that genetic changes have not yet accumulated in the control region.

### **Assessment of nested clade analysis (NCA)**

The NCA detected patterns of significant geographical associations among shy-type haplotypes that were generally similar to those revealed by AMOVA and  $F_{ST}$  estimates: results were highly significant when analysing between the two species (the level of the entire cladogram), whereas within-species clades were not supported statistically. The inference key predicted that shy albatrosses arose through range expansion via long distance colonisation by white-capped albatrosses. Such

expansion is supported by other evidence. White-capped albatross breeding islands are part of a geographical ‘hotspot’ of albatross diversity in New Zealand’s subantarctic, which is a likely source of progressive occupation of albatrosses throughout the Southern Ocean. Further, the breeding islands of all of the closest relatives of shy-types are geographically much nearer to white-capped albatross populations than to shy albatross populations. Several features of the haplotype network also suggest that shy albatrosses arose from white-capped albatrosses and imply a founder effect. First, eleven individuals share the most common haplotype in shy albatrosses whereas only five individuals share the most common haplotype in white-capped albatrosses. Second, haplotype diversity of shy albatrosses is much lower than that in white-capped albatrosses — 15 (in 30 individuals) compared to 22 (in 29 individuals). Third, the number of mutational steps connecting all shy albatross haplotypes is much less than the number connecting all white-capped albatross haplotypes (41 as compared to 64). Hence based on all data presented here, it is highly plausible that shy albatross populations are derived from white-capped albatross populations and were founded by a small number of individuals.

Recent evidence suggests that predictions made by the inference key need to be made with extreme caution. Knowles and Maddison (2002) used computer simulated populations to evaluate the accuracy with which NCA distinguished between recurrent gene flow and historical processes, and found that NCA did not adequately account for the stochasticity in the coalescent process to effectively distinguish between the two processes. Consequently it is critical to examine the results of the inference key in the context of other evidence and not to rule out other explanations for the observed patterns too hastily. Nonetheless, that range expansion by white-capped albatrosses founded shy albatrosses appears to be highly plausible.

## Taxonomy

Two reasons were originally given to justify species status for shy and white-capped albatrosses: breeding asynchrony and lack of overlap of wing morphometrics (Robertson & Nunn 1998; but see Double *et al.* in review). The taxonomic separation of Salvin's and Chatham albatrosses from each other and from shy-type albatrosses has not been contested, likely due to the morphological distinctiveness of these three groups. Based on my results, the taxonomic separation of shy and white-capped albatrosses from Salvin's and Chatham albatrosses is clearly justified, however the taxonomic classification within shy and white-capped albatrosses remains unresolved.

There are several different theories on what constitutes a species, with accompanying criteria aimed at making species definitions operational (e.g. Cracraft 1983; Davis & Nixon 1992; de Queiroz 1998; Mayr 1996; Templeton 1989; Wiley 1978).

According to the biological species concept (BSC; Mayr 1996) species status hinges on mechanisms that render groups of organisms reproductively isolated. Despite the caveat that shy and white-capped albatrosses are presumably not reproductively isolated in a physiological sense, the temporal separation of their mating seasons is considered a valid premating isolating mechanism (Templeton 1989) and morphological divergence is not a prerequisite. Therefore, they appear to qualify as separate species under the BSC. There are two modern versions of the phylogenetic species concept (PSC; e.g. Cracraft 1983; Donoghue 1985). One of these focuses on minimum diagnosable units, and defines a species as the smallest group of organisms diagnosable by a unique combination of character states (Cracraft 1983). The single fixed difference in the mitochondrial DNA of shy and white-capped albatrosses would be sufficient evidence of divergence to qualify them as separate species according to this definition. A different version of the PSC categorises monophyletic groups as species (Donoghue 1985), a criterion that precludes shy and white-capped albatrosses as separate species based on the data presented here. The notion of

monophyletic groups as species has led to the emergence of the genealogical species concept, which uses gene coalescence as the criterion for species recognition. A group of organisms qualifies as a species “if their genes coalesce more recently within the group than between any member of the group and any organisms outside the group” (Baum & Shaw 1995:296). Data from several genes are required to estimate coalescence times, which are not currently available for shy and white-capped albatrosses.

Many taxonomists use genetic distance estimates to deduce taxonomic status of equivocal groups. This usually involves comparisons of sequence divergence estimates between morphologically distinct or “good” species with those of questionable taxonomic status (e.g. Norman *et al.* 2002). A study of the control region of the black-browed albatross complex (*Thalassarche melanophrys* and *T. impavida*) identified three genetically distinct groups (Burg & Croxall 2001). Two distinct groups were found within *T. melanophrys* that were separated by 4% sequence divergence, and *T. impavida* was separated by each of the *T. melanophrys* groups by around 6-7% sequence divergence. Burg and Croxall (2001) concluded that these data were good support for species recognition for *T. impavida*, and that both groups within *T. melanophrys* may represent separate species. By this method, the level of sequence divergence between shy and white-capped albatrosses (1.8%) appears insufficient to suggest separate species status.

The abundance of discrepant theories and criteria about what defines a species presents a difficult challenge when making practical decisions about where species boundaries lie. The British Ornithologists’ Union recently developed guidelines for the application of species limits in avian taxonomy. Species rank is approved once taxa are sufficiently divergent to be deemed ‘diagnosable’ and if the possibility that their gene pools will merge in the future is reasonably precluded (Helbig *et al.* 2002). For allopatric taxa, species rank is considered warranted if they are fully diagnosable

in each of several discrete or continuously varying characters, which include morphological, molecular, or structural (e.g. foraging strategy) features (Helbig *et al.* 2002). Their criteria would consider the single fixed nucleotide difference between shy and white-capped albatrosses insufficient to satisfactorily eliminate doubt about their future integrity, hence separate species status would not be recognised.

Shy and white-capped albatrosses appear to be either newly diverged species or in the process of speciating, such that for some species concepts 'acceptable' species-level differences have not yet accumulated. Results of ongoing morphological and nuclear genetic studies on shy and white-capped albatrosses will determine whether they have any diagnostic morphological characters and will test for male-mediated gene flow; therefore at present I reserve judgement on their taxonomic status in favour of readdressing the issue when more information becomes available.

## References

- Alexander K, Robertson G, Gales R (1997) The incidental mortality of albatrosses in longline fisheries. A report on the workshop from the first international conference on the biology and conservation of albatrosses. Australian Antarctic Division, Tasmania, Hobart, Australia.
- Allende LM, Rubio I, Ruiz-del-Valle V, Guillen J, Martinez-Laso J, Lowy E, Varela P, Zamora J, Arnaiz-Villena A (2001) The Old World sparrows (genus *Passer*) phylogeography and their relative abundance of nuclear mtDNA pseudogenes. *Journal of Molecular Evolution*, **53**, 144-154.
- Avise JC, Nelson WS, Bowen BW, Walker D (2000) Phylogeography of colonially nesting seabirds, with special reference to global matrilineal patterns in the sooty tern (*Sterna fuscata*). *Molecular Ecology*, **9**, 1783-1792.



- Baker AJ, Marshall HD (1997) Mitochondrial control region sequences as tools for understanding evolution. In: *Avian Molecular Evolution and Systematics* (ed. Mindell DP), pp. 51-82. Academic Press, New York.
- Baum DA, Shaw KL (1995) Genealogical perspectives on the species problem. In: *Experimental and Molecular Approaches to Plant Biosystematics* (eds. Hoch PC, Stephenson AG), pp. 289-303. Missouri Botanical Garden, St. Louis.
- Berg T, Moum T, Johansen S (1995) Variable numbers of simple tandem repeats make birds of the order Ciconiiformes heteroplasmic in their mitochondrial genomes. *Current Genetics*, **27**, 257-262.
- Brothers N (1991) Albatross mortality and associated bait loss in the Japanese longline fishery in the Southern Ocean. *Biological Conservation*, **55**, 255-268.
- Brothers N, Gales R, Hedd A, Robertson G (1998) Foraging movements of the shy albatross *Diomedea cauta* breeding in Australia - implications for interactions with longline fisheries. *Ibis*, **140**, 446-457.
- Burg T (2000) *Genetic Analyses of Albatrosses: Mating Systems, Population Structure and Taxonomy*. PhD thesis, Cambridge University, UK.
- Burg TM, Croxall JP (2001) Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology*, **10**, 2647-2660.
- Clement M, Posada D, Crandall KA (2000) TCS: a computer program to estimate gene genealogies. *Molecular Ecology*, **9**, 1657-1659.
- Cracraft J (1983) Species concepts and speciation analysis. In: *Current Ornithology* (ed. Johnson RF), pp. 159-187. Plenum Press, New York.
- Crandall KA (1996) Multiple interspecies transmissions of human and simian t-cell leukemia/lymphoma virus type I sequences. *Molecular Biology & Evolution*, **13**, 115-131.
- Crochet PA, Bonhomme F, Lebreton JD (2000) Molecular phylogeny and plumage evolution in gulls (Larini). *Journal of Evolutionary Biology*, **13**, 47-57.

- Croxall JP (1998) Research and conservation: a future for albatrosses? In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 269-290. Surrey Beatty and Sons, Chipping Norton.
- Croxall JP, Gales R (1998) An assessment of the conservation status of albatrosses. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 46-66. Surrey Beatty and Sons, Chipping Norton.
- Davis JI, Nixon KC (1992) Populations, genetic variation, and the delimitation of phylogenetic species. *Systematic Biology*, **41**, 421-435.
- de Queiroz K (1998) The general lineage concept of species, species criteria, and the process of speciation. In: *Endless Forms: Species and Speciation* (eds. Howard DJ, Berlocher SH), pp. 57-75. Oxford University Press, Oxford.
- Donoghue MJ (1985) A critique of the biological species concept and recommendations for a phylogenetic alternative. *Bryologist*, **88**, 172-181.
- Double MC, Gales R, Reid T, Brothers N, Abbott CL (2003) Morphometric comparison of Australian shy and New Zealand white-capped albatrosses. *Emu*.
- Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics* **131**:479-491.
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-791.
- Fitzsimmons NN, Limpus CJ, Norman JA, Goldizen AR, Miller JD, Moritz C (1997) Philopatry of male marine turtles inferred from mitochondrial DNA markers. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 8912-8917.
- Gales R (1998) Albatross populations: status and threats. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 20-45. Surrey Beatty and Sons, Chipping Norton.

- Garnett S, Crowley G (2000) *The Action Plan for Australian Birds*. Environment Australia.
- Gomez-Zurita J, Petitpierre E, Juan C (2000) Nested cladistic analysis, phylogeography and speciation in the *Timarcha goettingensis* complex (Coleoptera, Chrysomelidae). *Molecular Ecology*, **9**, 557-570.
- Helbig AJ, Knox AG, Parkin DT, Sangster G, Collinson M (2002) Guidelines for assigning species rank. *Ibis*, **144**, 518-525.
- Holdaway RN, Worthy TH, Tennyson AJD (2001) A working list of breeding bird species of the New Zealand region at first human contact. *New Zealand Journal of Zoology* **28**, 119-187.
- Kidd MG, Friesen VL (1998) Sequence variation in the guillemot (Alcidae: *Cepphus*) mitochondrial control region and its nuclear homolog. *Molecular Biology & Evolution*, **15**, 61-70.
- Knowles LL, Maddison WP (2002) Statistical phylogeography. *Molecular Ecology*, **11**, 2623-2635.
- Kocher TD, Thomas WK, Meyer A, Edwards SV, Paabo S, Villablanca FX, Wilson AC (1989) Dynamics of mitochondrial DNA evolution in animals: amplification and sequencing with conserved primers. *Proceedings of the National Academy of Sciences of the United States of America*, **86**, 6196-6200.
- Mayr E (1996) What is a species, and what is not? *Philosophy of Science*, **63**, 262-277.
- Milot E, Gibbs HL, Hobson KA (2000) Phylogeography and genetic structure of northern populations of the yellow warbler (*Dendroica petechia*). *Molecular Ecology*, **9**, 667-681.
- Murray TE, Bartle JA, Kalish SR, Taylor PR (1993) Incidental capture of seabirds by Japanese southern bluefin tuna longline vessels in New Zealand waters, 1988-1992. *Bird Conservation International*, **3**, 181-210.

- Nei M, Li WH (1979) Mathematical model for studying genetic variation in terms of restriction endonucleases. *Proceedings of the National Academy of Sciences USA*, **76**, 5269-5273.
- Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, **31**, 165-176.
- Nielsen KK, Arctander P (2001) Recombination among multiple mitochondrial pseudogenes from a Passerine genus. *Molecular Phylogenetics & Evolution*, **18**, 362-369.
- Norman JA, Christidis L, Joseph L, Slikas B, Alpers D (2002) Unravelling a biogeographical knot: origin of the 'leapfrog' distribution pattern of Australo-Papuan sooty owls (Strigiformes) and logrunners (Passeriformes). *Proceedings of the Royal Society of London - Series B: Biological Sciences*, **269**, 2127-2133.
- Nunn GB, Cooper J, Jouventin P, Robertson CJR, Robertson GG (1996) Evolutionary relationships among extant albatrosses (Procellariiformes, Diomedidae) established from complete cytochrome-b gene sequences. *Auk*, **113**, 784-801.
- Nunn GB, Stanley SE (1998) Body size effects and rates of cytochrome b evolution in tube-nosed seabirds. *Molecular Biology and Evolution*, **15**, 1360-1371.
- Oyler-McCance SJ, Kahn NW, Burnham KP, Braun CE, Quinn TW (1999) A population genetic comparison of large- and small-bodied sage grouse in Colorado using microsatellite and mitochondrial DNA markers. *Molecular Ecology*, **8**, 1457-1465.
- Paulo OS, Jordan WC, Bruford MW, Nichols RA (2002) Using nested clade analysis to assess the history of colonization and the persistence of populations of an Iberian lizard. *Molecular Ecology*, **11**, 809-819.
- Perkins SL (2001) Phylogeography of Caribbean lizard malaria: tracing the history of vector-borne parasites. *Journal of Evolutionary Biology*, **14**, 34-45.

- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.
- Posada D, Crandall KA, Templeton AR (2000) GeoDis: a program for the cladistic nested analysis of the geographical distribution of genetic haplotypes. *Molecular Ecology*, **9**, 487-488.
- Robertson CJR, Nunn GB (1998) Towards a new taxonomy for albatrosses. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 13-19. Surrey Beatty and Sons, Chipping Norton.
- Ruokonen M, Kvist L, Lumme J (2000) Close relatedness between mitochondrial DNA from seven *Anser* goose species. *Journal of Evolutionary Biology*, **13**, 532-540.
- Ryan PG, Keith DG, Kroese M (2002) Seabird bycatch by tuna longline fisheries off southern Africa, 1998-2000. *South African Journal of Marine Science*, **24**, 103-110.
- Schneider S, Kueffer JM, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000. A Software Program for Population Genetic Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva.
- Shirihai H (2002) *A complete guide to Antarctic wildlife* Alula Press, Degerby, Finland.
- Simon C, Frati F, Beckenbach A, Crespi B, Liu H, Flook P (1994) Evolution, weighting, and phylogenetic utility of mitochondrial gene sequences and a compilation of conserved polymerase chain reaction primers. *Annals of the Entomological Society of America*, **87**, 651-701.
- Sorenson MD, Fleischer RC (1996) Multiple independent transpositions of mitochondrial DNA control region sequences to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 15239-15243.
- Swofford DL (2002) *PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods)*. Sinauer Associates, Sunderland, MA.

- Templeton AR (1989) The meaning of species and speciation: a genetic perspective. In: *Speciation and its Consequences* (eds. Otte D, Endler JA), pp. 3-27. Sinauer, Sunderland, MA.
- Templeton AR (1998) Nested clade analyses of phylogeographic data - testing hypotheses about gene flow and population history. *Molecular Ecology*, **7**, 381-397.
- Templeton AR, Boerwinkle E, Sing CF (1987) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. I. Basic theory and an analysis of alcohol dehydrogenase activity in *Drosophila*. *Genetics*, **117**, 343-352.
- Templeton AR, Routman E, Phillips CA (1995) Separating population structure from population history - a cladistic analysis of the geographical distribution of mitochondrial DNA haplotypes in the tiger salamander, *Ambystoma tigrinum*. *Genetics*, **140**, 767-782.
- Templeton AR, Sing CF (1993) A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping. IV. Nested analyses with cladogram uncertainty and recombination. *Genetics*, **134**, 659-669.
- Tennyson A (2002) A recent review of New Zealand seabirds. *Australasian Seabird Bulletin*, **38**, 3-5.
- Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignments through sequence weighting, position specific gap penalties and weight matrix choice. *Nucleic Acids Research*, **22**, 4673-4680.
- Tickell WLN (2000) *Albatrosses*. Pica Press, Sussex.
- Trueman JWH (1998) Reverse successive weighting. *Systematic Biology*, **47**, 733-737.

Wennerberg L (2001) Breeding origin and migration pattern of dunlin (*Calidris alpina*) revealed by mitochondrial DNA analysis. *Molecular Ecology*, **10**, 1111-1120.

Wiley EO (1978) The evolutionary species concept reconsidered. *Systematic Zoology*, **39**, 399-413.

Worheide G, Hooper JNA, Degnan BM (2002) Phylogeography of western Pacific *Leucetta 'chagosensis'* (Porifera: Calcarea) from ribosomal DNA sequences: implications for population history and conservation of the Great Barrier Reef World Heritage Area (Australia). *Molecular Ecology*, **11**, 1753-1768.



# Chapter II



Genetic structure, conservation genetics,  
and evidence of speciation by range  
expansion in shy and white-capped  
albatrosses

*Molecular Ecology (2003) 12: 2953-2962*



## Abstract

Six variable microsatellite loci were used to examine genetic structuring in the closely-related shy albatross (*Thalassarche cauta*) and white-capped albatross (*T. steadi*). First, levels of genetic differentiation between the species, and among three populations within each species, were analysed using AMOVA,  $F_{ST}$ , and  $R_{ST}$ . I found high levels of genetic structuring and detected many unshared alleles between the species, which provide strong evidence against any contemporary gene flow between them. Within each species, shy albatross populations were found to be genetically distinct whereas white-capped albatross populations were undifferentiated, which implies that dispersal events are much rarer in the former than in the latter. These results formed the basis for the recommendation that the three white-capped albatross populations (as a whole) and each shy albatross population be treated as separate units for conservation. Second, levels of genetic diversity and allelic patterns in shy and white-capped albatrosses were assessed for whether they support earlier mtDNA results suggesting that shy albatrosses arose through range expansion of white-capped albatrosses. All measures indicated lower genetic diversity within shy albatrosses than within white-capped albatrosses and upheld the hypothesis that shy albatrosses were founded by white-capped albatrosses.

## Introduction

There are several challenges associated with studying the dispersal behaviour of pelagic bird species. Their wide-ranging movements at-sea make them difficult to track in the wild, and their breeding locations are often sufficiently remote that extensive demographic studies are rare (but see Croxall *et al.* 1990; Prince *et al.* 1994; Weimerskirch *et al.* 1997; Weimerskirch & Jouventin 1998). For albatrosses, these challenges have resulted in relatively few empirical studies on their dispersal patterns, which have traditionally been limited to studies of banded populations. While satellite telemetry and data loggers have recently provided a wealth of new information on the at-sea behaviour of albatrosses (e.g. Arnould *et al.* 1996; Brothers *et al.* 1998; Sagar & Weimerskirch 1996; Weimerskirch & Guionnet 2002), these techniques are not well-suited to monitoring the movements of large groups, for which indirect inferences using genetic data can be very effective. Indeed, genetic techniques have been used to elucidate the dispersal patterns and/or migratory behaviour of several oceanic vertebrate species (e.g. Baker *et al.* 1994; Bass *et al.* 1996; Encalada *et al.* 1998; Fitzsimmons *et al.* 1997; Lahanas *et al.* 1998).

Despite their high dispersal potential due to inherently high vagility, studies of banded populations of albatrosses have shown them to be extremely philopatric (Prince *et al.* 1994; Sagar *et al.* 1998; Weimerskirch *et al.* 1985). This has led to predictions of restricted gene flow among breeding locations and to the growing view that many individual albatross breeding islands harbour distinct terminal taxa (Robertson & Nunn 1998). However a recent genetic study found little differentiation among populations of grey-headed albatross (*Thalassarche chrysostoma*) and described them as globally panmictic based on microsatellites and mitochondrial DNA (Burg & Croxall 2001). The same study reported contrasting levels of genetic differentiation among black-browed albatross (*T. melanophrys*) populations, with the Falkland Islands population found to be highly divergent from

a group of three otherwise undifferentiated, yet geographically distant, populations (Diego Ramirez, South Georgia, and Kerguelen; Burg & Croxall 2001).

Seabird mortality caused by drowning on longline fishing hooks is occurring throughout the Southern Ocean (Tuck *et al.* 2003) and is cited repeatedly as being responsible for population declines in several albatross species (Croxall *et al.* 1990; de la Mare & Kerry 1994; Nel *et al.* 2002; Prince *et al.* 1994; Weimerskirch *et al.* 1997; Weimerskirch & Jouventin 1987; Weimerskirch & Jouventin 1998). Despite having received considerably less recent attention than longlining bycatch, significant albatross mortality also results from interactions with trawl fisheries (Bartle 1991; Robertson *et al.* 2003). Deaths tend to occur when birds collide with trawl warps or other cables, or become entangled in the net itself (Baker *et al.* 2002). Thought to be largely a result of incidental fisheries-related deaths, 83% of albatross species are considered threatened (Croxall & Gales 1998).

Knowledge of the population genetic structure of threatened species can contribute substantially towards evaluating population viability (Haig 1998) and identifying appropriate conservation strategies, however this information is unavailable for most albatross species. A fundamental concept in conservation biology is that of the evolutionarily significant unit (ESU; reviewed in Fraser & Bernatchez 2001), developed to guide the prioritisation of units for conservation below taxonomic levels. Information on whether and how genetic diversity is partitioned among albatross populations could lead to the identification of units within species that are of particular conservation relevance, and thereby aid the development of management plans aimed at preserving current levels of genetic diversity.

Shy and white-capped albatrosses (*Thalassarche cauta* and *T. steadi*) are both categorised as vulnerable under the International Union for the Conservation of Nature criteria (Croxall & Gales 1998) and are thought to be killed on longline

fishing hooks in Australian (Brothers 1991), New Zealand (Murray *et al.* 1993), and South African (Ryan *et al.* 2002) waters, and by squid trawl fishing around New Zealand (Robertson *et al.* 2003). Previously classified as subspecies, their recent recognition as separate species followed a taxonomic revision suggested by Robertson and Nunn (1998), and has remained hotly contended. Indeed, their morphologies are extremely similar (Double *et al.* 2003) and the evolutionary relationship between them is yet to be resolved (Abbott & Double 2003 [Chapter I]). A recent study of the relationship between shy and white-capped albatrosses using mitochondrial control region sequence data found strong genetic structuring between them despite low sequence divergence (Abbott & Double 2003 [Chapter I]). The data also suggested shy albatrosses were founded through range expansion by white-capped albatrosses. Little genetic structuring was found within either species.

The current study uses microsatellite DNA markers to address two main aims. First, the hierarchical apportionment of genetic diversity within and between shy and white-capped albatrosses is examined, for which microsatellites have advantages over previously-used mtDNA. Bi-parental inheritance allows estimates of gene exchange as mediated by both sexes, and hypervariability provides high resolution of the spatial organisation of genetic variation within species. These data will assist in discerning the dispersal tendencies of shy and white-capped albatrosses and in the identification of evolutionarily significant units for conservation purposes. Second, I compare levels of genetic diversity between shy and white-capped albatrosses and assess whether they support the mtDNA hypothesis that shy albatrosses were founded through range expansion of white-capped albatrosses. Ultimately these data should also help resolve their taxonomic ambiguity.

## Methods

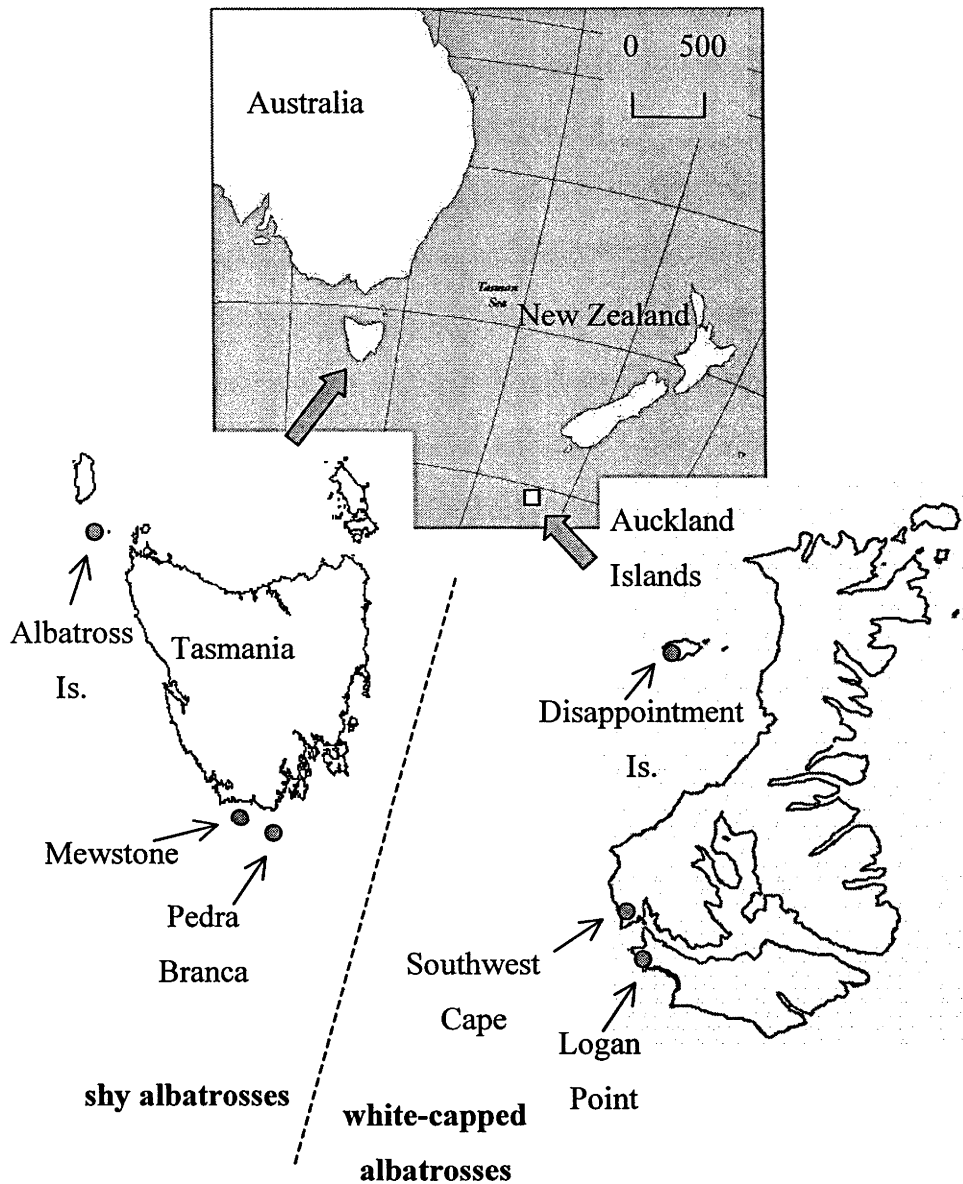
### Nomenclature

In accordance with the most recent taxonomic hypothesis for albatrosses I adopt the species nomenclature suggested by Robertson & Nunn (1998). I use the names 'shy albatross' and 'white-capped albatross' and refer to them as species, although I recognise that the appropriateness of this classification is uncertain.

### Study species and sample collection

Shy albatrosses breed exclusively on three islands near Tasmania, Australia, which is roughly 1800 km from the main breeding sites of white-capped albatrosses in the Auckland Islands group in New Zealand's subantarctic (Figure 1). A small population of 50-100 pairs of white-capped albatrosses breed at Bollons Island in the Antipodes Islands.

I obtained DNA by taking blood from nestlings at breeding colonies. The population sizes and relative locations of breeding sites, with the number of nestlings sampled indicated in parentheses, are as follows. Shy albatross populations at Mewstone ( $n = 23$ ) and Pedra Branca ( $n = 20$ ) are separated by about 50 km and have *c.* 7000 and *c.* 250 breeding pairs respectively (Brothers *et al.* 1998). Approximately 420 km away is a shy albatross population on Albatross Island ( $n = 24$ ) with *c.* 5000 pairs (Brothers *et al.* 1998). The three white-capped albatross breeding sites in the Auckland Islands are located within approximately 30 km of each other (population sizes taken from Gales 1998): *c.* 72000 pairs on Disappointment Island ( $n = 24$ ); *c.* 3000 pairs at Southwest Cape, Auckland Island ( $n = 23$ ); and *c.* 100 pairs at Logan Point, Adams Island ( $n = 23$ ). Samples from the small population of white-capped albatrosses at Bollons Island were not obtained. All samples from each site were



**Figure 1.** Locations of sampled breeding sites for shy and white-capped albatrosses. Sample sizes per population are as follows: Albatross Island ( $n = 20$ ), Mewstone ( $n = 20$ ), Pedra Branca ( $n = 20$ ), Disappointment Island ( $n = 20$ ), Southwest Cape ( $n = 19$ ), and Logan Point ( $n = 20$ ).

collected during the same breeding season, and since both species lay only one egg per year there was no possibility of sampling siblings. I also purposely sampled chicks that were distributed as evenly throughout the colony as possible to reduce the probability of sampling related individuals at adjacent nests. A minimum of five nests separated the nests of sampled chicks, except at Logan Point where small population size demanded more intensive sampling.

### DNA extraction and microsatellites

DNA was extracted using ammonium acetate as described in Nicholls *et al.* (2000). A total of six microsatellite DNA markers were used. Two loci were originally isolated in grey-headed albatrosses (*Thalassarche chrysostoma*) and one in wandering albatrosses (*Diomedea exulans*): Dc5, Dc9 (Burg 1999), and De35 (Burg & Croxall 2001) respectively. Three loci were isolated from shy albatrosses using both enriched and unenriched methods. An enriched library was made following procedures outlined in Paetkau (1999a) but yielded only one variable autosomal locus, Tc2 (Tc2F1: 5'-CAATGTGTTATTCTCTGTCC-3' and Tc2R1: 5'-CTATGCCAGTCACTGCATGG-3'). Locus Tc65 (Tc65F: 5'-ACTGGACTGCTTTCTTTCCG-3' and Tc65R: 5'-GACATTAAGTGGTGTTCCTTAGGG-3') was isolated following procedures described in Scott *et al.* (2001). Locus Tc9 (Tc9F1: 5'-ATACGCCGCACTTGATCC-3' and Tc9R4: 5'-CTCGCTACGACCGCATCC-3') was isolated following a similar approach to Dawson *et al.* (1997). Four variable Z-linked loci were also isolated (information available on request).

All but one loci were amplified using a single-reaction nested PCR method using forward primers with fluorescently labelled M13(-21) attached to their 5' ends as described in Schuelke (2000). Reverse primers were "PIG-tailed" to improve scoring (Brownstein *et al.* 1996).

PCR reactions were 10  $\mu$ l in volume. All loci except Tc65 were amplified using 50-100 ng of genomic DNA, 200  $\mu$ M dNTP, 2 pmol each of fluorescently labelled M13 primer and reverse primer, 0.2 pmol of M13-labelled forward primer, 2.3 mM  $\text{MgSO}_4$ , and 0.5 U Platinum *Taq* DNA polymerase (Invitrogen) in 1x PCR Amplification buffer and 1x PCR Enhancer Solution (Invitrogen). PCR cycles were 3 min at 94 °C; a 'touchdown' of 25 s at 94 °C, 25 s at 70 °C to 50 °C (dropping 5 °C per 3 cycles), 25 s at 72 °C; 30 cycles of 25 s at 94 °C, 25 s at 48 °C, 25 s at 72 °C and one final cycle of 3 min at 72 °C. Locus Tc65 was amplified using 50-100 ng of genomic DNA, 200  $\mu$ M dNTP, 4.3 mM  $\text{MgCl}_2$ , 2 pmol of fluorescently labelled forward primer, 2 pmol of reverse primer, 0.5 U of *AmpliTaq* DNA Polymerase (Applied Biosystems) in 1x reaction buffer. A 3-step cycling profile with annealing temperature of 50 °C was repeated 35 times. PCR products from all loci for each individual were combined, ethanol precipitated, and multiplexed in a single gel lane of a ABI Prism 377 automated sequencer. Data were analysed using GeneScan 3.1.2 (PE Biosystems).

## Analysis

Twenty to 24 individuals from each of the six main shy and white-capped albatross breeding islands (sample size per population listed in Table 1) were screened at six microsatellite loci. I tested for departures from Hardy-Weinberg equilibrium using Guo and Thompson's (1992) analogue of Fisher's Exact Test and for linkage disequilibrium using GENETPOP (Raymond & Rousset 1995).

An analysis of molecular variance (AMOVA, Excoffier *et al.* 1992) was performed in Arlequin (Schneider *et al.* 2000) using all six populations to estimate the total percentage variance attributable to between species differences, to between population differences within species, and to differences among individuals within populations. An AMOVA was also performed separately on shy and white-capped



**Table 1.** Estimates of the number of alleles per locus ( $N_a$ ) and the expected ( $H_E$ ) and observed ( $H_O$ ) heterozygosities for six microsatellite loci in three populations of shy albatross and three populations of white-capped albatross. Population names are abbreviated as follows: Albatross Island (AI), Mewstone (MW), Pedra Branca (PB), Southwest Cape (SW), Logan Point (LG), and Disappointment Island (DP). After Bonferroni correction, none of the  $H_E$  estimates showed a significant departure from Hardy-Weinberg equilibrium.

Population	Tc2	Tc65	Tc9	Dc5	Dc9	De35	Average
<b>Shy</b>							
AI ( $n = 24$ )							
$N_a$	3	4	5	2	3	7	4.00
$H_E$	0.62	0.70	0.61	0.37	0.52	0.82	0.61
$H_O$	0.58	0.75	0.63	0.42	0.33	0.75	0.58
MW ( $n = 23$ )							
$N_a$	3	5	5	2	3	7	4.17
$H_E$	0.63	0.75	0.61	0.52	0.20	0.80	0.59
$H_O$	0.61	0.83	0.65	0.43	0.17	0.70	0.57
PB ( $n = 20$ )							
$N_a$	3	3	5	2	3	8	4.00
$H_E$	0.35	0.55	0.50	0.34	0.32	0.78	0.47
$H_O$	0.35	0.70	0.60	0.25	0.20	0.75	0.48
<b>White-capped</b>							
SW ( $n = 23$ )							
$N_a$	5	6	8	3	5	10	6.17
$H_E$	0.60	0.72	0.80	0.46	0.40	0.89	0.65
$H_O$	0.65	0.78	0.78	0.43	0.43	0.87	0.66
LG ( $n = 23$ )							
$N_a$	4	7	6	2	4	10	5.50
$H_E$	0.37	0.76	0.74	0.44	0.55	0.87	0.62
$H_O$	0.26	0.70	0.70	0.17	0.61	0.91	0.56
DP ( $n = 24$ )							
$N_a$	7	7	7	3	5	10	6.50
$H_E$	0.60	0.75	0.85	0.42	0.59	0.87	0.68
$H_O$	0.50	0.67	0.88	0.46	0.58	0.92	0.67

albatrosses to partition within-species variance into among population and within population components. Population differentiation was estimated through pairwise population comparisons of  $F_{ST}$  (Wright 1978) and its analogue  $R_{ST}$  (Slatkin 1995) using Arlequin and RSTCALC (Goodman 1997) respectively. For a visual representation of genetic patterns, a principal co-ordinates (PCO) analysis of pairwise population  $F_{ST}$  values was performed using GenAEx V5.04 (Peakall & Smouse 2001).

Genetic diversity in shy and white-capped albatrosses was examined in the context of the mtDNA hypothesis that shy albatrosses were founded by a small number of white-capped albatrosses (Abbott & Double 2003 [Chapter I]). Genetic theory predicts a significant reduction in effective population size will cause a population to lose substantial genetic variation, corresponding to a reduction in allele number and heterozygosity at polymorphic loci (Chakraborty & Nei 1977; Nei *et al.* 1975). I pooled populations within each species and tested for species level differences of mean allelic diversity and mean heterozygosity per locus by a two-way analysis of variance with locus and population as main parameters using JMP Version 3.0 (SAS Institute Inc., Cary, NC, USA). I also examined the presence/absence of particular alleles at the species level, and used a one-way ANOVA to test for a difference between the mean frequency of white-capped albatross alleles also found in shy albatrosses and the mean frequency of alleles unique to white-capped albatrosses (as the putative 'source population').

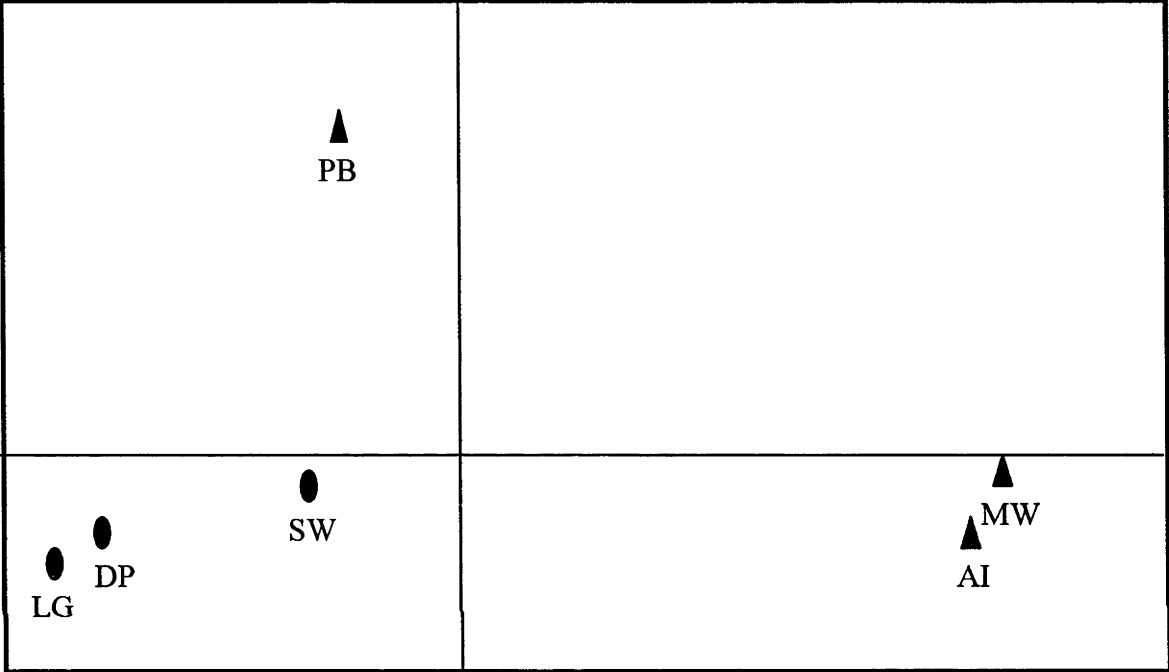
I also employed the technique of Cornuet and Luikart (1996) for detecting recent population bottlenecks using allele frequency data. The premise is that bottlenecked populations experience a transient phase whereby the observed heterozygosity is higher than the expected heterozygosity at mutation-drift equilibrium given the allele number, because allele number decreases more quickly than heterozygosity (Piry *et al.* 1999). A one-tailed Wilcoxon signed rank test implemented in the program

BOTTLENECK (Piry *et al.* 1999) was used to test for heterozygosity excess under two mutation models: the infinite alleles model (IAM) and the stepwise mutation model (SMM). Tests were performed separately on each population, and on the pooled data for each species.

## Results

The six microsatellite loci assayed had an overall mean heterozygosity across all samples and loci of 0.57, with allele numbers ranging from 3 to 10 alleles per locus (Table 1). Before sequential Bonferroni correction (Rice 1989), 2 of 36 (5.5%) locus-population comparisons showed significant departures from Hardy-Weinberg equilibrium (Logan Point at loci Tc2 and Dc5), whereas no departures were evident after the correction. All tests for linkage disequilibrium were not significant.

Not surprisingly, variability between species is usually expected to be higher than variability within species. However, a hierarchical AMOVA for shy and white-capped albatrosses showed that the between-species component of variance (3.9%) was lower than that among populations within species (4.7%). Subsequent AMOVA tests on each species separately indicated that among-population genetic variability was much higher within shy albatrosses (9.0%) than within white-capped albatrosses (1.2%). Further, the PCO analysis (Figure 2) illustrated that much of the genetic variance within shy albatrosses was attributable to high divergence of the Pedra Branca population. To test the effect of the Pedra Branca population on the hierarchical AMOVA I repeated the analysis excluding it, and this yielded more typical results: 6.3% of genetic variance was attributed to between-species differences, and 2.3% was attributed to among-population differences within species. Unexpectedly high divergence of the Pedra Branca population was not detected in an earlier study using mtDNA control region data (Abbott & Double 2003 [Chapter I]).



**Figure 2.** PCO plot of pairwise population  $F_{ST}$  values among three populations of shy albatrosses (Albatross Island (AI), Mewstone (MW), and Pedra Branca (PB); represented by triangles) and three populations of white-capped albatrosses (Southwest Cape (SW), Logan Point (LG), and Disappointment Island (DP); represented by ovals).

Strong genetic structuring between shy and white-capped albatrosses was indicated by highly significant estimates of  $F_{ST}$  (0.054,  $P < 0.001$ ) and  $R_{ST}$  (0.049,  $P < 0.001$ ). Similarly, all pairwise cross-species population comparisons showed high levels of genetic structuring (Table 2). Pairwise population  $F_{ST}$  comparisons within shy albatrosses showed highly significant structure ( $P < 0.001$ ) whereas only one pairwise population comparison within white-capped albatrosses was significant (Southwest Cape and Logan Point,  $P < 0.05$ ). Apart from a few discrepancies, results of pairwise population comparisons of  $R_{ST}$  were similar to those of  $F_{ST}$  but with lower significance levels (Table 2). The general consensus from significance patterns of  $F_{ST}$  and  $R_{ST}$  estimates depicts high levels of genetic structuring both between the two species and within shy albatrosses, and low levels of genetic structuring within white-capped albatrosses.

Across all loci, the total number of alleles and the number of species-specific alleles were both much higher in white-capped albatrosses than in shy albatrosses (Table 3). The mean number of alleles per locus was 4.1 for shy albatrosses and 6.1 for white-capped albatrosses, which was statistically different (ANOVA,  $F = 15.8$ ,  $P < 0.001$ ). Mean heterozygosity across all loci and all populations within species was 0.54 for shy albatrosses and 0.63 for white-capped albatrosses, although this difference was not significant (ANOVA,  $F = 2.3$ ,  $P = 0.07$ ).

Higher allelic diversity at microsatellite loci in white-capped albatrosses as compared to shy albatrosses is consistent with earlier mtDNA findings of higher haplotype diversity in the former than the latter (Abbott & Double 2003 [Chapter I]). Of the 46 alleles sampled in white-capped albatrosses, 21 were only sampled in this species and the remaining 25 were also sampled in shy albatrosses. I compared the allelic frequencies of the species-specific alleles in white-capped albatrosses with the frequencies of those that were shared with shy albatrosses for evidence that founder effects may have eliminated low frequency alleles in the latter. The mean frequency

**Table 2.** Matrix of pairwise comparisons of  $F_{ST}$  (Wright 1978; above diagonal) and  $R_{ST}$  (Slatkin 1995; below diagonal) for three shy albatross populations and three white-capped albatross populations. Asterisks denote significant values as follows: \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$ .

Species	Population	Shy albatrosses			White-capped albatrosses		
		Albatross	Mewstone	Pedra	S.W. Cape	Logan Pt.	Disappt.
Shy	Albatross	—	0.050***	0.119***	0.071***	0.097***	0.095***
	Mewtone	-0.001	—	0.108***	0.053***	0.114***	0.095***
	Pedra	0.088**	0.086**	—	0.060***	0.095***	0.084***
White-capped	S.W. Cape	0.079**	0.067**	0.005	—	0.016*	0.007
	Logan Pt.	0.053*	0.033	0.078**	0.027	—	0.012
	Disappt.	0.141***	0.118***	0.053*	-0.007	0.043*	—

**Table 3.** Individual alleles (symbolised by alphabetical characters), number of alleles (*Na*), and number of species-specific alleles for six microsatellite loci in white-capped (WC) and shy albatrosses.

Locus	Species	Alleles	<i>Na</i>	No. spp. specific alleles
Tc2	WC	ABCDEFG	7	4
	Shy	C EF	3	0
Tc65	WC	ABCDEFGHIJ	10	6
	Shy	A GHI K	5	1
Tc9	WC	ABCDEFG IJKL	11	6
	Shy	BCDEF H	6	1
Dc5	WC	ABC	3	1
	Shy	A C	2	0
Dc9	WC	ABCDE	5	2
	Shy	AB E	3	0
De35	WC	ABCDEFGHIJ	10	2
	Shy	ABCD F HIJK	9	1
Totals	WC		46	21
	Shy		28	3

of alleles in white-capped albatross that were also found in shy albatrosses (0.20) was much higher than the mean frequency of alleles unique to white-capped albatrosses (0.04), and this difference was significant (ANOVA,  $F = 32.7$ ,  $P < 0.001$ ). Further, the size distribution of alleles at each locus is much more continuous in white-capped albatrosses than in shy albatrosses (Table 3).

None of the BOTTLENECK tests for heterozygosity excess was significant. This may reflect insufficient statistical power because only six loci were used. A set of fewer than 20 polymorphic loci is considered small (Cornuet & Luikart 1996) for this application, and at least four variable loci are needed for “any possibility of obtaining significant ( $P < 0.05$ )” results (Piry *et al.* 1999:502).

## Discussion

Using six microsatellite loci, I found strong genetic structuring between shy and white-capped albatrosses and strong population differentiation within shy albatrosses. Especially high levels of differentiation were associated with the shy albatross population on Pedra Branca. Allelic diversity measures indicated lower genetic diversity within shy albatrosses than within white-capped albatrosses.

### Genetic structure in shy and white-capped albatrosses

High within-species heterozygosity associated with hypervariable markers tends to decrease the magnitude of differentiation estimates (Hedrick 1999), hence direct comparisons between  $F_{ST}$  values derived from mtDNA and nuclear microsatellites are inappropriate. However, the consensus on levels of genetic structuring between shy and white-capped albatrosses obtained from microsatellites corroborate Abbott & Double's (2003 [Chapter I]) findings using mitochondrial control region haplotypes.



Strong genetic differentiation between the species was indicated by highly significant  $F_{ST}$  and  $R_{ST}$  values, and by allelic patterns showing 24 species-specific alleles. These results resolutely suggest against contemporary gene flow between shy and white-capped albatrosses, and thereby confirm the demographic isolation of the two species.

Within-species patterns of genetic structuring were highly discrepant between shy and white-capped albatrosses. For shy albatrosses, AMOVA results showed a reasonably large and highly significant component of total variation residing among populations, and most  $F_{ST}$  and  $R_{ST}$  estimates between pairs of populations were highly significant. In essence the scenario was reversed for white-capped albatrosses. Although AMOVA attributed a small but significant component of total variation to among population variance,  $F_{ST}$  and  $R_{ST}$  estimates between pairs of white-capped albatross populations were mostly non significant. Thus it appears that while there is sufficient gene flow among white-capped albatross populations to prevent differentiation, gene flow among shy albatross populations is clearly limited.

I found the shy albatross population at Pedra Branca to be anomalous. Pedra Branca is less divergent from white-capped albatross populations than are the other shy albatross populations, and levels of divergence between Pedra Branca and both Albatross Island and Mewstone are notably higher than those between the latter two. There are two possible explanations for this. First, shy albatrosses at Pedra Branca may have a closer relationship to white-capped albatrosses than those breeding elsewhere, perhaps due to more recent colonisation or migration. Alternatively, the pattern could simply be a consequence of random genetic drift. Many factors implicate the latter scenario. First, the number of albatrosses breeding on Pedra Branca is relatively small which makes it more susceptible to genetic drift, and the island is densely occupied by a rapidly increasing population of Australasian gannets (*Morus serrator*; Bunce *et al.* 2002) such that inward migration of albatrosses seems

unlikely. Second, the frequencies of individual alleles at Pedra Branca are erratic with respect to whether they are more similar to those found in white-capped albatrosses or to those of other shy albatrosses, and for a few alleles are highly discrepant from both (data not shown), which may be suggestive of greater effects of drift. Further, the presence/absence patterns of alleles at Pedra Branca are highly consistent with those of the other two shy albatross populations and therefore do not implicate different patterns of contemporary or historical genetic exchange between Pedra Branca and white-capped albatrosses.

### **Inferences about dispersal**

There is a small but growing body of evidence that dispersal among albatross populations is not demographically negligible. For example, the wandering albatross (*Diomedea exulans*) was recently reported to have metapopulation dynamics such that the level of juvenile dispersal among populations was sufficient to influence population dynamics at geographically distant breeding islands (Inchausti & Weimerskirch 2002). Similarly, as outlined earlier, five grey-headed albatross populations with a circumpolar distribution around Antarctica were described as genetically panmictic (Burg & Croxall 2001), thereby suggesting consequential levels of inter-island migration.

Results presented here imply the possibility of isolation by distance within shy and white-capped albatrosses. The white-capped albatross populations at the Auckland Islands are relatively close to one another geographically and are exchanging enough migrants to prevent the accumulation of genetic differences among them. In contrast, migration is presumably much rarer among the genetically distinct shy albatross populations, which are more distantly located from one another except for Mewstone and Pedra Branca. However as mentioned earlier, the genetic isolation of Pedra Branca may be augmented by other factors. Insight into the possibility of

isolation by distance would be gained by investigating the genetic relationship between white-capped albatrosses at the Auckland Islands and those found about 1200 km away at Bollons Island, which were not sampled here. A general trend of isolation by distance was found between two distinct groups of wandering albatross species (*Diomedea exulans*, *D. gibsoni*, and *D. antipodensis*) based on mtDNA sequences (Burg 2000). Two distinct groups were found: those from the Atlantic or Indian Oceans and those from New Zealand. Sequence divergence within groups was 1.8% and 1.0% respectively, and that between groups was 5.2%.

### Conservation implications

The concept of targeting conservation priorities below taxonomic levels to preserve diversity within species is well accepted, and lead to the emergence of operational units for conservation called ‘evolutionarily significant units’ (ESUs). However the definition of an ESU is hotly debated (e.g. Crandall *et al.* 2000; Dimmick *et al.* 1999; Moritz 1999; Paetkau 1999b; Pennock & Dimmick 1997; Vogler & Desalle 1994; Waples 1998), largely due to discordant species concepts underlying ESU definitions (Fraser & Bernatchez 2001). There are also practical difficulties associated with the implementation of any one ESU concept because varying circumstances influence their efficacy given their inherent strengths and weaknesses (examples in Fraser & Bernatchez 2001). Fraser and Bernatchez (2001) recommend flexibility around designating ESU status and support a unifying conceptual framework of ‘adaptive evolutionary conservation’ (AEC) to mitigate these difficulties, under the premise that ESU concepts are not fundamentally irreconcilable. The general definition of an ESU under AEC is “a lineage demonstrating highly restricted gene flow from other such lineages”, and is satisfied by any criteria that signal lineage sorting through highly reduced gene flow (Fraser & Bernatchez 2001).

According to this ESU definition, the fixed difference in mtDNA between shy and white-capped albatrosses and the highly divergent patterns of allele frequencies at microsatellite loci clearly confirm separate ESU status for each species. Information on ecological differences between the species is generally unavailable – while they breed asynchronously [their breeding seasons start about two months apart (Robertson & Nunn 1998)], whether this is a heritable trait representing an adaptive change or an environmentally based difference is unknown. Genetic data do not reject the null hypothesis that the three white-capped albatross breeding sites at the Auckland Islands represent a single population, however as recommended by Taylor and Dizon (1996), given the absence of demographic information about population dynamics in this species I refrain from declaring them a homogeneous group. In contrast, although all shy albatross populations appear to belong to a single evolutionary lineage, and thereby are not ESUs under the AEC definition, sufficient divergence was found to reject the hypothesis that they should be considered genetically equivalent for conservation. It may be useful to consider them management units (MU), a term introduced by Moritz (1994) to define “shallow” population genetic subdivisions that are the most logical units for population monitoring.

### **Inferences about speciation processes**

I compared levels of genetic diversity between shy and white-capped albatrosses to assess the mtDNA hypothesis that shy albatrosses were founded by white-capped albatrosses (Abbott & Double 2003 [Chapter I]). A significant reduction in effective population size is expected to correspond to a reduction in allele number and heterozygosity at variable loci (Chakraborty & Nei 1977; Nei *et al.* 1975). Allelic diversity is thought to be a more sensitive indicator of a bottleneck because low frequency alleles from a source population are easily eliminated in a small founded population by random genetic drift (Nei *et al.* 1975). These predictions have been

supported empirically, with low genetic diversity attributed to bottlenecks and/or founder events in several species (e.g. Bouzat *et al.* 1998; Comstock *et al.* 2002; Hoelzel *et al.* 1993; Houlden *et al.* 1996; O'Brien *et al.* 1987; Taylor *et al.* 1994).

My finding of lower genetic diversity in shy albatrosses than white-capped albatrosses, and supporting allele frequency data, uphold the hypothesis suggested by Abbott & Double (2003 [Chapter I]) that shy albatrosses were founded by a small number of white-capped albatrosses. Allelic diversity and heterozygosity are both lower in shy albatrosses than white-capped albatrosses, though only the first is statistically significant. With few exceptions, shy albatross alleles represent a subset of total alleles found in white-capped albatrosses at each locus, with low frequency alleles in white-capped albatrosses less likely to be observed in shy albatrosses than higher frequency alleles. It is likely that some low frequency alleles in white-capped albatrosses were not represented in the founding genotypes, and others were eliminated post-colonisation by genetic drift. The three alleles unique to shy albatrosses had low frequencies (from 0.021 to 0.087), and may reflect new mutations in shy albatrosses or may be present but undetected in white-capped albatrosses and therefore represent sampling artefacts.

Bottleneck effects are most drastic when the founding population size is very small and diminish rapidly as bottleneck severity lessens (Nei *et al.* 1975). Clegg *et al.* (2002) estimated that island populations of silvereyes (*Zosterops lateralis*) were founded by flocks of about 100 individuals, which was sufficiently large to prevent severe founder effects. The readily perceptible genetic signature of a population bottleneck in shy albatrosses suggests that they were colonised by a small number of individuals, which is the most plausible scenario for albatrosses for other reasons. As discussed earlier, most individual albatrosses breed in their natal colony which leaves few dispersing individuals available to act as founders. Further, mindful that some small populations of albatrosses are remnants after population decline, there

are several island populations with one to five breeding pairs (Gales 1998) that may represent recent colonisation events. Small populations are highly prone to extinction due to stochastic effects, thus most of these colonisation attempts are expected to fail.

My results are concordant with earlier mtDNA results (Abbott & Double 2003 [Chapter I]) indicating the absence of contemporary intermigration between shy and white-capped albatrosses and thus confirms their demographic independence. Dispersal events appear sufficiently rare in shy albatrosses to allow genetic divergence among populations, and sufficiently common in white-capped albatrosses to prevent it. This pattern of genetic structuring formed the basis for the recommendation that the three white-capped albatross populations (as a whole) and each shy albatross population be treated as separate units for conservation. Results presented here uphold the hypothesis that shy albatrosses arose through range expansion by white-capped albatrosses (Abbott & Double 2003 [Chapter I]). Together these studies provide the first molecular evidence of a colonisation event that lead to a speciation event in albatrosses.

## References

- Abbott CL, Double MC (2003) Phylogeography of shy and white-capped albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy. *Molecular Ecology*, **12**, 2747-2758.
- Arnould JPY, Briggs DR, Croxall JP, Prince PA, Wood AG (1996) The foraging behaviour and energetics of wandering albatrosses brooding chicks. *Antarctic Science*, **8**, 229-236.

- Baker CS, Slade RW, Bannister JL, *et al.* (1994) Hierarchical structure of mitochondrial DNA gene flow among humpback whales *Megaptera novaeangliae*, world-wide. *Molecular Ecology*, **3**, 313-327.
- Baker GB, Gales R, Hamilton S, Wilkinson V (2002) Albatrosses and petrels in Australia: a review of their conservation and management. *Emu*, **102**, 71-97.
- Bartle JA (1991) Incidental capture of seabirds in the New Zealand subantarctic squid trawl fishery, 1990. *Bird Conservation International*, **1**, 351-359.
- Bass AL, Good DA, Bjorndal KA, *et al.* (1996) Testing models of female reproductive migratory behaviour and population structure in the Caribbean hawksbill turtle, *Eretmochelys imbricata*, with mtDNA sequences. *Molecular Ecology*, **5**, 321-328.
- Bouzat JL, Cheng HH, Lewin HA, *et al.* (1998) Genetic evaluation of a demographic bottleneck in the greater prairie chicken. *Conservation Biology*, **12**, 836-843.
- Brothers N (1991) Albatross mortality and associated bait loss in the Japanese longline fishery in the Southern Ocean. *Biological Conservation*, **55**, 255-268.
- Brothers N, Gales R, Hedd A, Robertson G (1998) Foraging movements of the shy albatross *Diomedea cauta* breeding in Australia - implications for interactions with longline fisheries. *Ibis*, **140**, 446-457.
- Brownstein MJ, Carpten JD, Smith JR (1996) Modulation of non-templated nucleotide addition by *Taq* DNA polymerase - primer modifications that facilitate genotyping. *Biotechniques*, **20**, 1004-1010.
- Bunce A, Norman FI, Brothers N, Gales R (2002) Long-term trends in the Australasian gannet (*Morus serrator*) population in Australia: the effect of climate change and commercial fisheries. *Marine Biology*, **141**, 263-269.
- Burg T (2000) *Genetic Analyses of Albatrosses: Mating Systems, Population Structure and Taxonomy*. PhD thesis, Cambridge University, UK.
- Burg TM (1999) Isolation and characterization of microsatellites in albatrosses. *Molecular Ecology*, **8**, 338-341.

- Burg TM, Croxall JP (2001) Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology*, **10**, 2647-2660.
- Chakraborty R, Nei M (1977) Bottleneck effects on average heterozygosity and genetic distance with the stepwise mutation model. *Evolution*, **31**, 347-356.
- Clegg SM, Degnan SM, Kikkawa J, *et al.* (2002) Genetic consequences of sequential founder events by an island-colonizing bird. *Proceedings of the National Academy of Sciences of the United States of America*, **99**, 8127-8132.
- Comstock KE, Georgiadis N, Pecon-Slattery J, *et al.* (2002) Patterns of molecular genetic variation among African elephant populations. *Molecular Ecology*, **11**, 2489-2498.
- Cornuet JM, Luikart G (1996) Description and power analysis of two tests for detecting recent population bottlenecks from allele frequency data. *Genetics*, **144**, 2001-2014.
- Crandall KA, Bininda-Emonds ORP, Mace GM, Wayne RK (2000) Considering evolutionary processes in conservation biology. *Trends in Ecology & Evolution*, **15**, 290-295.
- Croxall JP, Gales R (1998) An assessment of the conservation status of albatrosses. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 46-66. Surrey Beatty & Sons, Chipping Norton.
- Croxall JP, Rothery P, Pickering SPC, Prince PA (1990) Reproductive performance, recruitment and survival of wandering albatrosses *Diomedea exulans* at Bird Island, South Georgia. *Journal of Animal Ecology*, **59**, 775-795.
- Dawson RJG, Gibbs HL, Hobson KA, Yezerinac SM (1997) Isolation of microsatellite DNA markers from a passerine bird, *Dendroica petechia* (the yellow warbler), and their use in population studies. *Heredity*, **79**, 506-514.
- de la Mare WK, Kerry KR (1994) Population dynamics of the wandering albatross (*Diomedea exulans*) on Macquarie Island and the effects of mortality from longline fishing. *Polar Biology*, **14**, 231-241.



- Dimmick WW, Ghedotti MJ, Grose MJ, *et al.* (1999) The importance of systematic biology in defining units of conservation. *Conservation Biology*, **13**, 653-660.
- Double MC, Gales R, Reid T, Brothers N, Abbott CL (2003) Morphometric comparison of Australian shy and New Zealand white-capped albatrosses. *Emu*, **103**, 287-294.
- Encalada SE, Bjørndal KA, Bolten AB, *et al.* (1998) Population structure of loggerhead turtle (*Caretta caretta*) nesting colonies in the Atlantic and Mediterranean as inferred from mitochondrial DNA control region sequences. *Marine Biology*, **130**, 567-575.
- Excoffier L, Smouse P, Quattro J (1992) Analysis of molecular variance inferred from metric distances among DNA haplotypes: application to human mitochondrial DNA restriction data. *Genetics*, **131**, 479-491.
- Fitzsimmons NN, Limpus CJ, Norman JA, *et al.* (1997) Philopatry of male marine turtles inferred from mitochondrial DNA markers. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 8912-8917.
- Fraser DJ, Bernatchez L (2001) Adaptive evolutionary conservation: towards a unified concept for defining conservation units. *Molecular Ecology*, **10**, 2741-2752.
- Goodman SJ (1997) Rst calc - a collection of computer programs for calculating estimates of genetic differentiation from microsatellite data and determining their significance. *Molecular Ecology*, **6**, 881-885.
- Guo S, Thompson E (1992) Performing the exact test of Hardy-Weinberg proportion from multiple alleles. *Biometrics*, **48**, 361-372.
- Haig SM (1998) Molecular contributions to conservation. *Ecology*, **79**, 413-425.
- Hedrick PW (1999) Highly variable loci and their interpretation in evolution and conservation. *Evolution*, **53**, 313-318.

- Hoelzel AR, Halley J, Obrien SJ, *et al.* (1993) Elephant seal genetic variation and the use of simulation models to investigate historical population bottlenecks. *Journal of Heredity*, **84**, 443-449.
- Houlden BA, England PR, Taylor AC, Greville WD, Sherwin WB (1996) Low genetic variability of the koala *Phascolarctos cinereus* in south-eastern Australia following a severe population bottleneck. *Molecular Ecology*, **5**, 269-281.
- Inchausti P, Weimerskirch H (2002) Dispersal and metapopulation dynamics of an oceanic seabird, the wandering albatross, and its consequences for its response to long-line fisheries. *Journal of Animal Ecology*, **71**, 765-770.
- Lahanas PN, Bjorndal KA, Bolten AB, *et al.* (1998) Genetic composition of a green turtle (*Chelonia mydas*) feeding ground population - evidence for multiple origins. *Marine Biology*, **130**, 345-352.
- Moritz C (1994) Defining evolutionarily significant units for conservation. *Trends in Ecology & Evolution*, **9**, 373-375.
- Moritz C (1999) A molecular perspective on the conservation of biodiversity. In: *The Biology of Biodiversity* (ed. Kato S), pp. 21-34. Springer Verlag, Tokyo.
- Murray TE, Bartle JA, Kalish SR, Taylor PR (1993) Incidental capture of seabirds by Japanese southern bluefin tuna longline vessels in New Zealand waters, 1988-1992. *Bird Conservation International*, **3**, 181-210.
- Nei M, Maruyama T, Chakraborty R (1975) The bottleneck effect and genetic variability in populations. *Evolution*, **29**, 1-10.
- Nel DC, Ryan PG, Crawford RJM, Cooper J, Huyser OAW (2002) Population trends of albatrosses and petrels at sub-Antarctic Marion Island. *Polar Biology*, **25**, 81-89.
- Nicholls JA, Double MC, Rowell DM, Magrath RD (2000) The evolution of cooperative and pair breeding in thornbills *Acanthiza* (Pardalotidae). *Journal of Avian Biology*, **31**, 165-176.

- O'Brien SJ, Wildt DE, Bush M, *et al.* (1987) East African cheetahs: evidence for two population bottlenecks? *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 508-511.
- Paetkau D (1999a) Microsatellites obtained using strand extension: an enrichment protocol. *Biotechniques*, **26**, 690-697.
- Paetkau D (1999b) Using genetics to identify intraspecific conservation units: a critique of current methods. *Conservation Biology*, **13**, 1507-1509.
- Peakall R, Smouse PE (2001) GenAlEx V5.04: *Genetic Analysis in Excel. Population Genetic Software for Teaching and Research*. Australian National University, Canberra, Australia. Available at: <http://www.anu.edu.au/BoZo/GenAlEx/>.
- Pennock DS, Dimmick WW (1997) Critique of the evolutionarily significant unit as a definition for distinct population segments under the U.S. endangered species act. *Conservation Biology*, **11**, 611-619.
- Piry S, Luikart G, Cornuet JM (1999) BOTTLENECK: a computer program for detecting recent reductions in the effective population size using allele frequency data. *Journal of Heredity*, **90**, 502-503.
- Prince PA, Rothery P, Croxall JP, Wood AG (1994) Population dynamics of black-browed and grey-headed albatrosses *Diomedea melanophris* and *D. chrysostoma* at Bird Island, South Georgia. *Ibis*, **136**, 50-71.
- Raymond M, Rousset F (1995) GENEPOP version 1.2: population genetics software for exact tests and ecumenicism. *Journal of Heredity*, **86**, 248-249.
- Rice WR (1989) Analyzing tables of statistical tests. *Evolution*, **43**, 223-225.
- Robertson CJR, Bell E, Schofield P (2003) Autopsy report for seabirds killed and returned from New Zealand fisheries, 1 October 2001 to 30 September 2002. Department of Conservation, Wellington, New Zealand.
- Robertson CJR, Nunn GB (1998) Towards a new taxonomy for albatrosses. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 13-19. Surrey Beatty & Sons, Chipping Norton.

- Ryan PG, Keith DG, Kroese M (2002) Seabird bycatch by tuna longline fisheries off southern Africa, 1998-2000. *South African Journal of Marine Science*, **24**, 103-110.
- Sagar PM, Stahl JC, Molloy J (1998) Sex determination and natal philopatry of southern Buller's mollymawks (*Diomedea bulleri bulleri*). *Notornis*, **45**, 271-278.
- Sagar PM, Weimerskirch H (1996) Satellite tracking of southern Buller's albatrosses from The Snares, New Zealand. *Condor*, **98**, 649-652.
- Schneider S, Kueffer JM, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000. A Software Program for Population Genetic Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva.
- Schuelke M (2000) An economic method for the fluorescent labeling of PCR fragments. *Nature Biotechnology*, **18**, 233-234.
- Scott IAW, Hayes CM, Keogh JS, Morrison SF (2001) Isolation and characterization of novel microsatellite markers from the Australian water skink *Eulamprus kosciuskoi* and cross-species amplification in other members of the species-group. *Molecular Ecology Notes*, **1**, 28-30.
- Slatkin M (1995) A measure of population subdivision based on microsatellite allele frequencies. *Genetics*, **139**, 457-462.
- Taylor AC, Sherwin WB, Wayne RK (1994) Genetic variation of microsatellite loci in a bottlenecked species - the northern hairy-nosed wombat *Lasiorninus krefftii*. *Molecular Ecology*, **3**, 277-290.
- Taylor BL, Dizon AE (1996) The need to estimate power to link genetics and demography for conservation. *Conservation Biology*, **10**, 661-664.
- Tuck GN, Polacheck T, Bulman CM (2003) Spatio-temporal trends of longline fishing effort in the Southern Ocean and implications for seabird bycatch. *Biological Conservation*, **114**, 1-27.
- Vogler AP, Desalle R (1994) Diagnosing units of conservation management. *Conservation Biology*, **8**, 354-363.

- Waples RS (1998) Evolutionarily significant units, distinct population segments, and the endangered species act - reply to Pennock and Dimmick. *Conservation Biology*, **12**, 718-721.
- Weimerskirch H, Brothers N, Jouventin P (1997) Population dynamics of wandering albatross *Diomedea exulans* and Amsterdam albatross *D. amsterdamensis* in the Indian ocean and their relationships with long-line fisheries - conservation implications. *Biological Conservation*, **79**, 257-270.
- Weimerskirch H, Guionnet T (2002) Comparative activity pattern during foraging of four albatross species. *Ibis*, **144**, 40-50.
- Weimerskirch H, Jouventin P (1987) Population dynamics of the wandering albatross, *Diomedea exulans*, of the Crozet Islands: causes and consequences of the population decline. *Oikos*, **49**, 315-322.
- Weimerskirch H, Jouventin P (1998) Changes in population sizes and demographic parameters of six albatross species breeding on the French sub-Antarctic islands. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 84-91. Surrey Beatty and Sons, Chipping Norton.
- Weimerskirch H, Jouventin P, Mouglin JL, Stahl JC, Van Beveren M (1985) Banding recoveries and the dispersal of seabirds breeding in French austral and Antarctic territories. *Emu*, **85**, 22-33.
- Wright JS (1978) *Evolution and the Genetics of Natural Populations*. Vol. 4. *Variability Within and Among Natural Populations*, University of Chicago Press, Chicago.



# Chapter III



Molecular provenance analysis for shy  
and white-capped albatrosses killed by  
fisheries interactions in Australia, New  
Zealand and South Africa

## Abstract

Shy and white-capped albatrosses, *Thalassarche cauta* and *T. steadi* respectively, are phenotypically similar and are both thought to be suffering severe fisheries-related bycatch mortality in Australian, New Zealand, and South African waters.

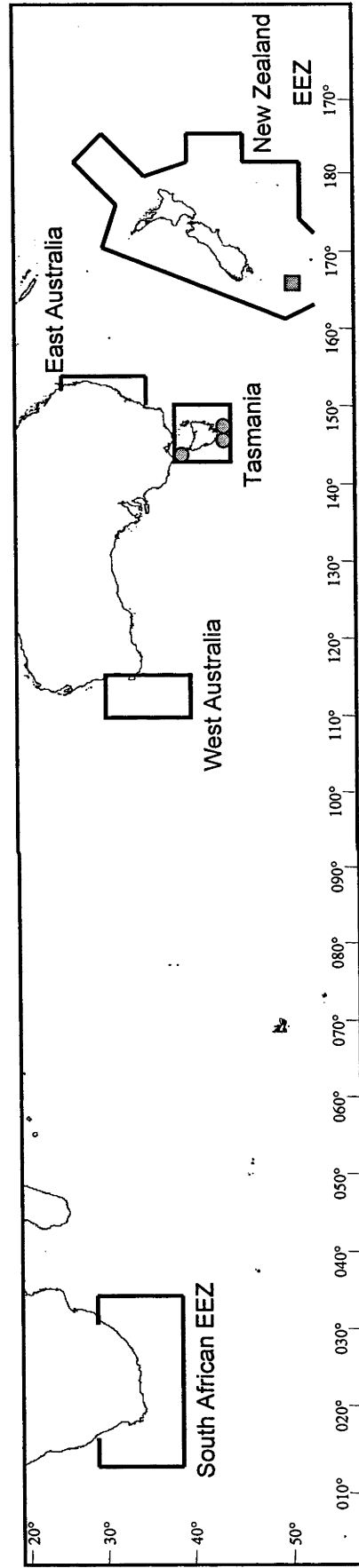
Assessments of the extent or scale of impact of bycatch mortality on these species have previously been precluded by difficulties identifying bycatch carcasses to species level. In this study a fast and simple molecular test based on a single nucleotide polymorphism in mtDNA of shy and white-capped albatrosses was used to determine the species composition of fisheries bycatch carcasses recovered from these regions. The accuracy of this test was found to be 98%, as evaluated using a more rigorous microsatellite-based method of provenance assignment. The only area where bycatch mortality of both species co-occurred was in Tasmanian waters. In all other zones the bycatch was exclusively comprised of white-capped albatrosses. Genotypic provenance assignment testing correctly assigned 72% of shy albatrosses to their population of origin. These data are the first to provide insight into the relative vulnerability of shy and white-capped albatrosses to bycatch mortality across a spatial scale, and to establish the vast differences in their at-sea distributions.

## Introduction

Albatross mortality arising from incidental interactions with fisheries is occurring on a global scale and is thought to affect a large proportion of species (Croxall *et al.* 1998; Gales 1998; Inchausti & Weimerskirch 2001; Nel *et al.* 2002a; Prince *et al.* 1998; Prince *et al.* 1994; Weimerskirch *et al.* 1997). Data collected by government observers aboard fishing vessels were instrumental in the initial discovery of the magnitude of the problem in the early 1990s (Brothers 1991; Murray *et al.* 1993) and since then have continued to provide estimates of bycatch rates (Gales *et al.* 1998; Klaer & Polacheck 1997; Nel *et al.* 2002b; Robertson *et al.* 2003; Ryan *et al.* 2002). However, phenotypic similarity amongst many albatross species and damage to some bycatch carcasses combine to make accurate identification of bycatch birds problematic. Species-specific bycatch information is lacking for species that are difficult to separate using morphometric characteristics. Further, population level differences in dispersal and migration patterns (e.g. Brothers *et al.* 1997) may cause variation in the susceptibility of individuals within species to bycatch mortality, and yet population-specific bycatch estimates have not been established for any albatrosses. This information is essential to investigate the impact of fisheries bycatch mortality on individual albatross species.

Shy and white-capped albatrosses, *Thalassarche cauta* and *T. steadi* respectively, are very closely-related taxa (Abbott & Double 2003a [Chapter II]) that are phenotypically similar (Double *et al.* 2003), hence are collectively referred to in this paper as 'shy-type' albatrosses. Shy albatrosses breed exclusively on three islands near Tasmania, Australia, which are roughly 1800 km from the three main breeding sites of white-capped albatrosses within the Auckland Islands group in New Zealand's subantarctic (Figure 1). Their global population sizes are estimated at 12000 and 75000 breeding pairs respectively (Gales 1998). Both species are considered vulnerable under the IUCN criteria (Croxall & Gales 1998) and are prone





**Figure 1.** Locations of the five geographic regions from which fisheries bycatch, live, and washed-up shy-type albatrosses were sampled. The main breeding site of white-capped albatrosses at the Auckland Islands, New Zealand, is marked by a grey square, and the three breeding sites of shy albatrosses near Tasmania, Australia, are marked with grey circles. The small population of white-capped albatrosses within the Antipodes Island group was not sampled.

to fisheries-related bycatch mortality in waters off Australia (Brothers 1991; Gales *et al.* 1998), New Zealand (Murray *et al.* 1993; Robertson *et al.* 2003), and South Africa (Ryan *et al.* 2002). Estimates of longlining bycatch from pelagic tuna fisheries in Australian waters in 1992 indicate that shy-type albatrosses were caught in the greatest numbers, comprising 49% of the total albatross catch (Klaer & Polacheck 1997). Similarly, the abundance of shy-type albatrosses killed by tuna longliners in South African waters from 1998-2000 was higher than that of any other albatross species (Ryan *et al.* 2002). In New Zealand, more shy-type albatrosses were killed by interactions with the squid trawl fishery between 1996 and 2001 than any other seabird (Robertson *et al.* 2003).

Assessments of the extent or scale of impact of bycatch mortality on each species have previously been precluded by difficulties identifying bycatch shy-type albatross carcasses to species level. Species-specific bycatch predictions cannot be made based on catch location data because the extent of foraging range overlap between shy and white-capped albatrosses is poorly known. While a discriminant function analysis using three morphological measurements can separate shy and white-capped albatrosses with up to 89% accuracy (Double *et al.* 2003), species identification is hindered because carcasses are often incomplete or in poor condition. Importantly, the recent recommendation that shy and white-capped albatrosses be considered separate Evolutionarily Significant Units for conservation purposes (Abbott & Double 2003a [Chapter II]) makes species-specific bycatch information especially critical. This recommendation was based on strong levels of genetic differentiation between the two species detected using both nuclear microsatellite markers (Abbott & Double 2003a [Chapter II]) and mitochondrial control region haplotypes (Abbott & Double 2003b [Chapter I]).

In contrast to morphologically-based methods of species identification, molecular methods can be time- and cost-effective and bear the logistical advantage of not

being dependent on the recovery of whole, undamaged bycatch carcasses. They have been widely used for the rapid identification of morphologically similar species (e.g. Dalebout *et al.* 1998; Greig *et al.* 2002; Shivji *et al.* 2002). Although to my knowledge genetic methods have not previously been used to identify albatross bycatch carcasses, they could assist substantially in the correct identification of many phenotypically similar species for which morphological species identification is highly error-prone. A molecular method of separating shy and white-capped albatrosses was recently developed by Abbott & Double (2003b [Chapter I]) that now provides a simple option for identifying shy-type albatross bycatch birds to species level.

Shy albatrosses are very well studied relative to most other seabirds due to a long-term demographic study that has been underway for over 20 years (Baker *et al.* 2002). Information is available on their foraging behaviour (Hedd & Gales 2001; Hedd *et al.* 1997), at-sea distribution (Brothers *et al.* 1998; Brothers *et al.* 1997; Hedd *et al.* 2001), and breeding biology (Gales 1993). Fisheries observer programs have also provided vital preliminary data on spatial and temporal variation of catch rates for shy albatrosses in Australia (Gales *et al.* 1998; Klaer & Polacheck 1997), although the lack of information on the population of origin of bycatch birds has precluded assessments of the potential impact on individual populations. This information is critical, as satellite telemetry data indicate differences in the foraging zones of the three island populations of shy albatrosses (Brothers *et al.* 1998), so bycatch mortality could affect populations differently. Further, genetic differences among their three populations have been found, so individual island populations have been recommended as the most logical units for population monitoring (Abbott & Double 2003a [Chapter II]). In contrast to this, the three white-capped albatross populations at the Auckland Islands were not found to be genetically divergent, a difference that may reflect isolation by distance as the white-capped albatross populations are located much more closely to one another than are shy albatross

populations (Abbott & Double 2003a [Chapter II]). Little else is known about white-capped albatrosses, including their breeding biology, population demographics, and at-sea distribution.

The main aim of this study was to use a molecular test to determine the species composition of bycatch shy-type albatross carcasses recovered from fishing vessels in Australian, New Zealand, and South African Exclusive Economic Zones, as these zones encompass all continental shelf regions thought to be regularly visited by these species. These data were subsequently used to identify geographic regions of relatively high risk of mortality for each species, and to establish whether there were age- or sex- ratio biases among bycatch birds for each species in each geographic zone. Results presented here provide valuable information about the relative vulnerability of shy and white-capped albatrosses to bycatch mortality across a spatial scale. Further, they provide novel information about the at-sea distributions of both species and enable the first evaluation of the extent of foraging range overlap between them. The secondary aim of this study was to obtain population-specific bycatch data for shy albatrosses using a genotype-based method of assigning provenance to bycatch birds. Ultimately these data may substantially assist the development of effective conservation strategies for both species, and will highlight the potential contribution of molecular markers to the species identification and provenance assignment of seabirds killed by fisheries activity.

## **Methods**

### **Sample collection at breeding colonies**

Known-provenance DNA samples were obtained by taking blood from nestlings at shy and white-capped albatross breeding colonies, which are located near Tasmania,

Australia, and in New Zealand's subantarctic respectively. The population sizes and relative locations of breeding sites, with the number of nestlings sampled indicated in parentheses, are as follows. Shy albatross populations at Mewstone ( $n = 23$ ) and Pedra Branca ( $n = 20$ ) are separated by about 50 km and have *c.* 7000 and *c.* 250 breeding pairs respectively (Brothers *et al.* 1998). Approximately 420 km away is a shy albatross population on Albatross Island ( $n = 24$ ) with *c.* 5000 pairs (Brothers *et al.* 1998). The three white-capped albatross breeding sites in the Auckland Islands are located within approximately 30 km of each other (population sizes taken from Gales 1998): *c.* 72000 pairs on Disappointment Island ( $n = 24$ ); *c.* 3000 pairs at Southwest Cape, Auckland Island ( $n = 23$ ); and *c.* 100 pairs at Logan Point, Adams Island ( $n = 23$ ). Samples from the small population ( $n = 50$ -100 pairs) of white-capped albatrosses located within the Antipodes Island group were not obtained. All samples from each site were collected during the same breeding season, and since both species lay only one egg per year there was no possibility of sampling siblings. I also purposely sampled chicks that were distributed as evenly throughout the colony as possible to reduce the probability of sampling related individuals at adjacent nests.

### **Sample collection at sea: bycatch and live birds**

Tissue samples were obtained from shy-type albatrosses killed by interactions with various fisheries (Table 1) in Australian, New Zealand, and South African waters (Figure 1). Specific catch date and location information were available for most samples. All bycatch birds collected from New Zealand and South Africa were caught within the Exclusive Economic Zones (EEZ) of these countries, which includes all waters within 200 nautical miles of the coast. Most (97%,  $n = 100$ ) bycatch birds collected from Australia were caught within Australia's EEZ, the exceptions being three birds caught in waters bordering this zone off Western Australia. Shy-type albatrosses are known to occur in high numbers in each of the

**Table 1.** Fisheries bycatch shy-type albatrosses recovered from New Zealand, South African, Tasmanian, Western Australian, and Eastern Australian waters that were analysed in this study. Total number of samples, the fishery they were caught by, and when they were collected are indicated.

Area	Fishery	Years sampled	<i>n</i> Samples
New Zealand	Squid Trawl	2000-2001	67
	Fish Trawl	1999-2001	10
	Tuna Longline	2000	3
South Africa	Tuna Longline	1999-2002	24
Tasmania	Tuna Longline	1988-2000	93
West Australia	Tuna Longline	1992-1996	6
East Australia	Tuna Longline	1991	1

sampled fishing zones, and are essentially absent from the Patagonian Shelf (Croxall & Wood 2002) and Humboldt Current regions off South America (Steve Howell & Larry Spear, pers. comm.) that are heavily exploited by other albatross species. Hence I believe my sampling is generally inclusive of all continental shelf regions regularly visited by these species. Further, although these data are not known for white-capped albatrosses, shy albatrosses are thought to be exclusively neritic feeders (Hedd *et al.* 2001) and are therefore not predicted to interact heavily with fishing vessels operating on the high seas.

Blood samples were also obtained from 18 live shy-type albatrosses caught (and subsequently released) over shelf waters off East Australia (35°S) between 1994 and 2000. This region was not well represented in the bycatch samples due to low fishing effort during the sampling period, however shy-type albatrosses are known to occur regularly there. Also included were tissue samples from four shy-type albatrosses that washed up on Fraser Island, Queensland (25°S) between 1995 and 2002.

Samples collected from Australian waters were stratified into one of three discrete geographic regions (Figure 1). The Western Australian region was comprised of six bycatch specimens caught between 30 – 40°S and 100 – 115°E, the Tasmanian region was comprised of 93 bycatch specimens caught between 39 – 44.5°S and 143 – 151°E, and the East Australian region was comprised of one bycatch specimen (caught at 36°S, 151°E) and 23 live-caught or washed up specimens as previously described.

### **Molecular species identification using mtDNA**

Molecular species identification was performed using a simple, PCR and restriction digest based, single nucleotide polymorphism (SNP) test as described by Abbott &

Double (2003b [Chapter I]). The test is based on a nucleotide substitution in Domain I of the mitochondrial control region of shy and white-capped albatrosses.

### **Species identification and provenance assignment using microsatellites**

A microsatellite based provenance assignment test was used to evaluate the accuracy of the mtDNA SNP test in separating shy and white-capped albatrosses. It was also attempted to identify the island of origin of bycatch shy albatrosses, as unlike white-capped albatrosses, their populations are genetically differentiated (Abbott & Double 2003a [Chapter II]). All known-origin shy and white-capped albatrosses were genotyped at six, previously-described, microsatellite markers (Abbott & Double 2003a [Chapter II]), as were all bycatch samples identified as shy albatrosses using the mtDNA SNP test. Assignment testing followed the Bayesian approach of Rannala and Mountain (1997) as implemented in GENECLASS 1.0.02 (Cornuet *et al.* 1999), whereby allele frequencies were used to calculate the likelihood of an individual's genotype occurring in each potential source population. Direct assignment and exclusion methods were employed. The direct assignment method assigned the individual to the population where its likelihood of belonging was highest. The exclusion method randomly generated simulated populations of 10 000 individuals for each source population based on sampled allele frequencies. The probability of belonging to each potential population was then calculated by comparing an individual's assignment likelihood to the distribution of assignment likelihoods of simulated individuals in each population. Individuals were assigned to all populations for which their probability of belonging to those populations exceeded 5%. Self-assignment of known-origin individuals was used to evaluate the performance of the tests before they were applied to bycatch birds. The 'leave one out' option was activated in self-assignments to avoid introducing bias associated with including the genotype of the individual to be assigned in estimating its source population's allele frequencies.



### **Age-class and sex of bycatch birds**

Age- and sex- ratios of bycatch birds for each species in each geographic zone were recorded. The age of birds was classified as either adult (i.e. breeding age) or sub-adult as this distinction is clear based on bill colouration, as described in Double *et al.* (2003). Sexes were determined by autopsy or by molecular sexing. For the latter, a 260 bp region on each sex chromosome was PCR amplified using primers P2 and P3 from Griffiths & Tiwari (1995). PCR reactions were 10 µl in volume and contained 50-100 ng of genomic DNA, 200 µM dNTP, 2 pmol of each primer, 3.2 mM MgCl<sub>2</sub>, and 0.5 U of *AmpliTaq* DNA Polymerase (Applied Biosystems) in 1x Opti-Prime PCR buffer (Stratagene). PCR cycles were 3 min at 94 °C; 35 cycles of 25 s at 94 °C, 25 s at 55 °C, and 25 s at 72 °C; and one final cycle of 3 min at 72 °C. PCR products were digested with 2U of *HaeIII*, and run through a 3% agarose gel. Three-band and two-band patterns were generated for females and males respectively.

## **Results**

### **Accuracy of mtDNA SNP test and genotypic assignment for species identification**

The direct assignment method was highly effective at separating shy and white-capped albatrosses: 98% (134/137) of known-origin birds sampled at their breeding colonies were correctly identified to species level during self-assignment. The three incorrect assignments were all white-capped albatrosses misidentified as shy albatrosses.

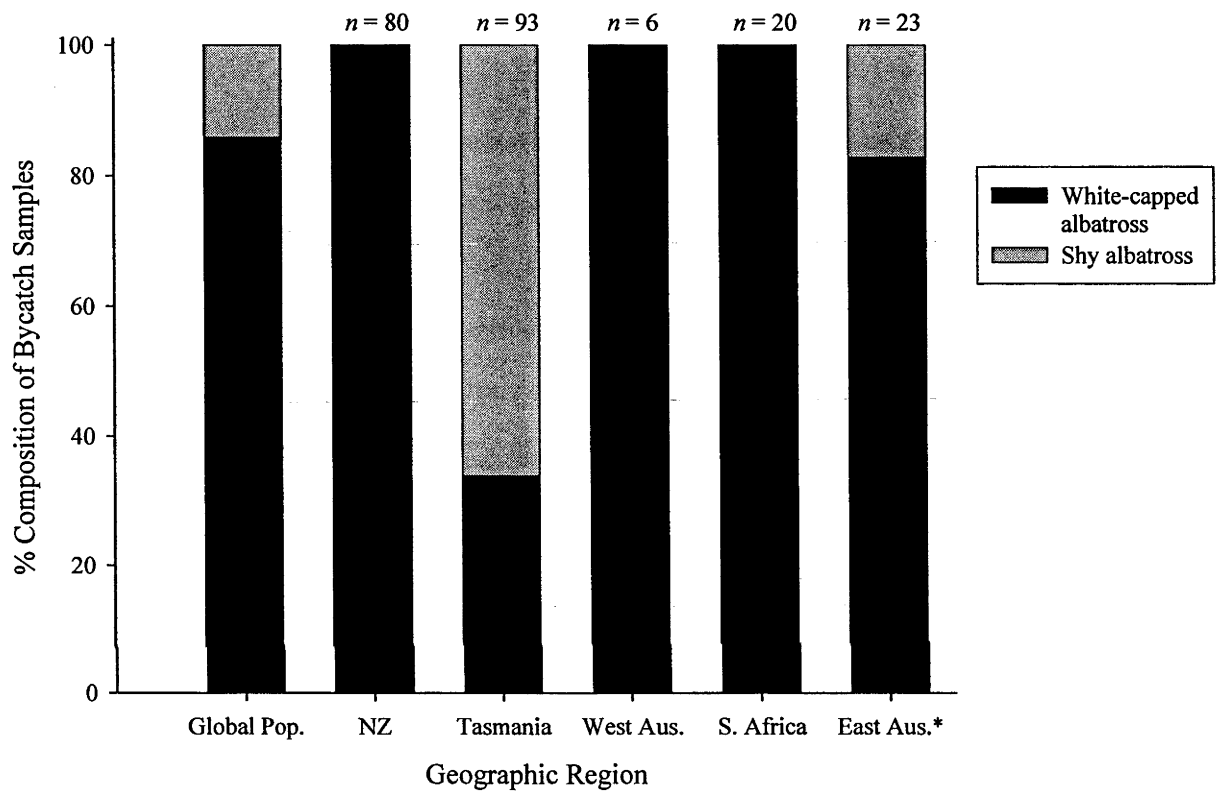
A total of 64 bycatch birds were identified as shy albatrosses by the mtDNA SNP test and were then used for genotypic assignment testing. Using the exclusion method, three of these unexpectedly generated assignment probabilities less than 0.0001 for all shy albatross populations, and each were subsequently found to have between two and four alleles specific to white-capped albatrosses (see Abbott & Double 2003a [Chapter II]). Hence these samples were re-classified as white-capped albatrosses. When this result is considered together with the 60 known-origin shy and white-capped albatross samples that were all identified correctly by the SNP test in an early study (Abbott & Double 2003b [Chapter I]), the accuracy of the test is estimated as 98% (121/124). The most credible explanation for the few misclassifications is that the 'diagnostic' site used in the SNP test to discriminate between the two species is not 100% fixed in white-capped albatrosses, as mtDNA haplotype diversity is much higher in white-capped albatrosses than in shy albatrosses (Abbott & Double 2003b [Chapter I]).

### **Species composition of shy-type albatross bycatch**

After correcting the species classification of the three bycatch white-capped albatrosses that had been misidentified as shy albatrosses by the mtDNA SNP test (described above), of 204 bycatch shy-type albatrosses sampled from Australian, New Zealand, and South African fishing zones, 70% (143 of 204) were identified as white-capped albatrosses and 30% (61 of 204) were identified as shy albatrosses.

The species composition of shy-type albatross samples obtained from New Zealand, South African, Tasmanian, Western Australian, and East Australian waters is shown in Figure 2. Bycatch mortality of shy albatrosses only occurred in Tasmanian waters, where this species comprised 66% ( $n = 93$ ) of total shy-type albatross bycatch.

White-capped albatrosses were killed in all areas, comprising 100% of the bycatch



**Figure 2.** Species composition of shy-type albatrosses sampled from New Zealand, Tasmania, Western Australia, South Africa, and East Australia. The global species composition based on the number of breeding pairs of each species is given for comparative purposes. \* All samples are bycatch birds except for the sample from East Australia, where only one is a known bycatch bird.

from New Zealand ( $n = 80$ ), South Africa ( $n = 24$ ) and West Australia ( $n = 6$ ), and 34% ( $n = 93$ ) of the bycatch caught off Tasmania.

The age-class composition and the monthly distributions of catch date for bycatch shy and white-capped albatrosses sampled across geographic regions are shown in Table 2. Shy albatrosses were caught in Tasmania during most months of the year and were biased towards adults (47 adults, 14 sub-adults;  $\chi^2$ : 17.9; df: 1;  $p \leq 0.001$ ). White-capped albatrosses caught in New Zealand were virtually all adults (78 adults, 2 sub-adults;  $\chi^2$ : 72.2; df: 1;  $p \leq 0.001$ ) and were caught during eight months of the year, with the vast majority caught from January to March, which is a time when the squid trawl fishery is known to catch large numbers of this species (Robertson *et al.* 2003). White-capped albatrosses were caught in Tasmania over eight calendar months and exhibited no age bias (12 adults, 20 sub-adults;  $\chi^2$ : 2.0; df: 1;  $p = 0.16$ ). The predominantly non-bycatch white-capped albatrosses sampled off East Australia were all caught between June and October and were comprised of approximately equal proportions of adults and sub-adults (8 adults, 11 sub-adults;  $\chi^2$ : 0.5; df: 1;  $p = 0.49$ ). All six white-capped albatrosses caught off Western Australia and all four shy albatrosses sampled off East Australia were sub-adults caught between June and August. Carcass-specific age data were not available for birds caught off South Africa, but have been recently described for these species in this region by Ryan *et al.* (2002).

A statistically significant sex difference was detected among white-capped albatrosses caught in Tasmania (10 males, 22 females;  $\chi^2$ : 4.5; df: 1;  $p \leq 0.05$ ), but not for those caught in New Zealand (43 males, 37 females;  $\chi^2$ : 0.5; df: 1;  $p = 0.50$ ) or South Africa (11 males, 13 females;  $\chi^2$ : 0.2; df: 1;  $p = 0.68$ ). When total bycatch across geographic zones was considered, sex-ratio biases were not evident for shy (24 males, 37 females;  $\chi^2$ : 2.8; df: 1;  $p = 0.1$ ) or white-capped (67 males, 75 females;  $\chi^2$ : 0.5; df: 1;  $p = 0.50$ ) albatrosses.

**Table 2.** Age and monthly distribution of catch date for shy and white-capped albatrosses caught in New Zealand (NZ), South Africa (SA), Tasmanian (Tas), Western Australian (WA), and East Australian (EA) waters. Catch date was not available for all samples analysed in this study.

Species	Area	n total	% Adult	Month											
				Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Shy	NZ	0													
	SA	0													
	Tas	61	77	1			2	3	10	11	2	18	6	8	
	WA	0													
	EA*	4	0						1	1	2				
White-capped	NZ	80	98	10	38	19	2		3	2		4	2		
	SA	24	44^	2				9			2	4	2	3	
	Tas	32	38	4		2	2	2	11	4			4	3	
	WA	6	0							3	3				
	EA*	19	42						3	2	8	1	5		

\* All samples are bycatch birds except for in East Australia, where the only bycatch bird was a white-capped albatross caught in August.  
^ Taken from Ryan *et al.* (2002)

### **Provenance assignment of bycatch shy albatrosses**

A full summary of results of genotypic assignment testing for shy albatrosses is presented in Table 3. Of 67 known-origin shy albatrosses self-assigned to their three potential source populations using the direct assignment method, 72% were correctly assigned. Using the exclusion method 15 (22%) were assigned to a single population, 11 (73%) of which were assigned correctly. Subsequent provenance assignment of 61 bycatch shy albatrosses using the direct method assigned 36 birds to Mewstone, 15 to Pedra Branca, and 10 to Albatross Island. The exclusion method assigned 13 of the 61 bycatch birds to a single population: 12 to Mewstone and one to Pedra Branca.

### **Discussion**

Earlier studies have shown strong levels of genetic differentiation between shy and white-capped albatrosses using both nuclear microsatellite markers (Abbott & Double 2003a [Chapter II]) and mitochondrial control region haplotypes (Abbott & Double 2003b [Chapter I]). The present study indicates that an mtDNA SNP test and a genotypic assignment test are both highly effective at separating shy and white-capped albatrosses, and thereby provides further evidence of the unequivocal genetic distinctiveness of these species. Species-specific information on the at-sea distribution of these species and their relative vulnerability to bycatch mortality across a spatial scale is established here. In addition, partial success is achieved in developing a genotypic assignment test to identify the provenance of shy albatrosses.

**Table 3.** Results of genotypic provenance assignment testing for known-origin and bycatch shy albatrosses using direct and exclusion assignment methods. Numbers of correctly assigned birds during self-assignment are highlighted in bold.

Assignment method	Population	<i>n</i>	<i>n</i> unambiguously assigned to:		
			Albatross	Mewstone	Pedra
Direct					
1. Self-assignment	Albatross I.	24	<b>16</b>	6	2
	Mewstone	23	5	<b>16</b>	2
	Pedra	20	1	1	<b>18</b>
2. Bycatch	unknown	61	10	36	15
Exclusion ( <i>p</i> ≥ 0.05)					
1. Self-assignment	Albatross I.	24	<b>5</b>	2	0
	Mewstone	23	0	<b>5</b>	0
	Pedra	20	1	1	<b>1</b>
2. Bycatch	unknown	61	0	12	1

### **Inferences about the at-sea distributions of shy and white-capped albatrosses**

Bycatch data can be used to confirm the presence of shy and white-capped albatrosses within a certain fishing zone and time frame, and to infer the relative degree to which different sexes and age-classes are being caught. However, because they are confounded by variation in the spatial and temporal distributions of fishing effort, observer coverage, and the at-sea distribution of the birds, and by whether any particular groups of birds are 'easier' to catch than others, they cannot be used to infer the absence of, or variation in, species abundance or to identify factors causing age or sex-related biases. Nonetheless, results presented here conclusively indicate vastly different at-sea distributions for shy and white-capped albatrosses.

Shy albatrosses were detected in Tasmanian and East Australian waters only. Adult shy albatrosses were detected exclusively in their 'local' waters around Tasmania, whereas sub-adults were detected in both Tasmanian and East Australia. These findings corroborate the current notion that adult shy albatrosses rely heavily, or perhaps even exclusively, on food resources close to their breeding colonies once they reach breeding age (Brothers *et al.* 1998). Studies using band recoveries, colour marking, and satellite telemetry found adult shy albatrosses to occur only in Tasmanian and nearby waters off Southern and Eastern Australia throughout the entire annual cycle (Brothers *et al.* 1998; Brothers *et al.* 1997; Hedd *et al.* 2001). In contrast to this, and despite absence of detection in the present study, banded sub-adult shy albatrosses have been recovered to at least some extent in all of the zones studied here (Brothers *et al.* 1997). Of 131 band recoveries of sub-adult shy albatrosses, 81 were recovered off Tasmania/Victoria, 23 off South Australia, nine off Western Australia, nine off New South Wales, eight off South Africa, and one off New Zealand (Brothers *et al.* 1997). That sub-adult shy albatrosses were not found further afield than East Australia in this study is presumably a reflection of the vastly greater abundance of white-capped albatrosses in these regions and correspondingly



insufficient sample sizes to detect them. However, because systematic collection of bycatch carcasses across a wide geographic range is logistically very difficult, the sampling done in this study is unlikely to be improved upon. Importantly, satellite telemetry is currently being undertaken to better resolve the at-sea distribution of sub-adult shy albatrosses (Birdlife International; <http://www.birdlife.net/index.html>), although small sample sizes are inevitable in such studies.

White-capped albatrosses were present in all zones sampled here. Adult white-capped albatrosses were detected in their 'local' New Zealand waters during most months of the year, but were also found in substantial numbers in Tasmanian and East Australian waters. Earlier research indicates that 44% of shy-type albatrosses found in South African waters (Ryan *et al.* 2002) are adults, which are presumably white-capped albatrosses based on the species composition data presented here. Sub-adult white-capped albatrosses were present in large proportions in all sampled regions except New Zealand, where they were rarely caught. This may be because most bycatch samples analysed from New Zealand were obtained from the squid trawl fishery that operates when breeding white-capped albatrosses are incubating and provisioning chicks (Gales 1998), which may be a time when they limit the length and therefore geographic range of their foraging trips. To my knowledge there have been no detailed studies on the at-sea distribution of white-capped albatrosses.

### **Vulnerability of shy and white-capped albatrosses to bycatch mortality**

The present study is the first to provide much-needed species-specific information on fisheries bycatch mortality for shy and white-capped albatrosses. Bycatch white-capped albatrosses were found to greatly outnumber bycatch shy albatrosses in all sampled areas outside of Tasmania. This finding is consistent with the fact that the global population size of white-capped albatrosses is much greater than for shy

albatrosses, and is not surprising given that shy-type albatrosses are caught in high numbers relative to other albatross species in each sampled area (Gales *et al.* 1998; Robertson *et al.* 2003; Ryan *et al.* 2002).

Results presented here depict drastically different degrees of vulnerability to bycatch mortality between shy and white-capped albatrosses. The range of adult shy albatrosses appears to be confined to waters around Tasmania and south eastern Australia, which is where most of an estimated 900 shy-type albatrosses were killed by Japanese tuna longliners between 1988 and 1997 (Gales *et al.* 1998). This is the time frame during which most of the samples analysed here were collected (Table 1), and my results indicate that the majority (~ 65%) of these birds were shy albatrosses. Since then however, tuna pelagic longlining effort in southern Australian (including Tasmanian) waters has changed dramatically; Japanese effort within Australian waters ceased in 1997 and current domestic effort in eastern Australia is concentrated in northern waters where the likelihood of encountering albatrosses is much lower. In addition, to my knowledge there are no other fisheries operating in Tasmanian waters that are known to kill shy-type albatrosses, hence adults of this species do not currently appear to be at risk. This is not true for sub-adult shy albatrosses, whose wider range (Brothers *et al.* 1997) likely brings them into contact with other fisheries that are known to catch albatrosses (see Tuck *et al.* 2003).

The vulnerability to fisheries bycatch mortality appears to be much greater for white-capped albatrosses (both adult and sub-adult) than for shy albatrosses as they occur over a much wider geographic range throughout their lives. While the previously described northward shift of pelagic longlining effort in Australian waters currently precludes any interaction between this fishery and white-capped albatrosses around Tasmania, fishing effort in other relevant fisheries continues to be high in most other areas where this species occurs. White-capped albatrosses accounted for all bycatch shy-type albatrosses analysed from South African waters, where pelagic longline

fishing effort increased from 0.23 million hooks in 1998 to almost 1.5 million hooks in 2000 (Tuck *et al.* 2003). Similarly, the squid trawl fishery operating south of New Zealand, in which the large majority of white-capped albatross bycatch analysed from the New Zealand EEZ were caught, continues to operate annually.

### **Genotypic provenance assignment testing**

In this study, shy albatross bycatch birds sampled from Australian waters were predominantly collected during a period when longline fishing effort overlapped to a higher extent with the foraging ranges of birds from the two southern populations (Mewstone and Pedra Branca) than the northern population (Albatross Island; Brothers *et al.* 1998). Of the southern populations, Mewstone has a much larger population size than Pedra Branca, which led to the prediction that Mewstone birds were most vulnerable to bycatch mortality (Brothers *et al.* 1998). Indeed, both assignment methods showed Mewstone birds to be suffering more bycatch mortality than birds from the other two shy albatross populations. This was despite that an overestimate of the number of bycatch birds from Albatross Island and Pedra Branca was expected, because neither the northern and therefore less vulnerable location of the former, or the small population size of the latter could be factored into the assignment tests. Thus genetic analyses of bycatch samples presented here, in combination with data on the at-sea distribution of each shy albatross population and the distribution of fishing effort, strongly imply that the Mewstone population was impacted most severely by fishing mortality off Tasmania at the time sampling occurred. Importantly however, as explained earlier, recent changes in the geographic distribution of pelagic longlining effort in Australian waters have eliminated overlap between this fishery and shy albatrosses.

Several factors affect the accuracy of assignment testing, including the time of divergence of populations, the sample size of source populations, and the number of

loci used (Cornuet *et al.* 1999). Other studies using genotypic assignment testing obtained assignment rates of 60% in polar bears (*Ursus maritimus*; Paetkau *et al.* 1995), 28-85% (median 61%) across ten taxonomically diverse species (Manel *et al.* 2002), 90% in brown trout (*Salmo trutta*; Hansen *et al.* 2000) and 93-100% in rock-wallabies (*Petrogale lateralis*; Eldridge *et al.* 2001). It is likely that divergence time is a major factor affecting assignment probabilities in shy albatrosses, as the  $F_{ST}$  estimate of 0.05 between Albatross Island and Mewstone (Abbott & Double 2003a [Chapter II]), which is where approximately 98% of shy albatrosses breed, may be insufficient to obtain greater assignment rates. Divergence time is known to highly influence false-inclusion errors, which were prevalent here (data not shown), and are characterised by at least one incorrect population being included along with the correct population as the possible source population (Cornuet *et al.* 1999). Despite this, it may be possible to improve the performance of provenance assignment for shy albatrosses by increasing the source population sample sizes and the number of loci used (Cornuet *et al.* 1999).

Further studies are needed to better understand the conservation implications of fisheries bycatch mortality on shy and white-capped albatrosses. These include research into current levels of fishing effort in all major fisheries known to kill shy-type albatrosses in each region. Behavioural and demographic data on scarcely studied white-capped albatrosses are also urgently needed. Knowledge of temporal and spatial variation in their at-sea distribution would help identify seasons and/or geographic regions of particularly high risk of fisheries-related mortality, and information on the status of white-capped albatross populations and their breeding biology, including confirmation of whether they are annual or biennial breeders, will greatly assist population viability assessments for this species.

## References

- Abbott CL, Double MC (2003a) Genetic structure, conservation genetics, and evidence of speciation by range expansion in shy and white-capped albatrosses. *Molecular Ecology*, **12**, 2953-2962.
- Abbott CL, Double MC (2003b) Phylogeography of shy and white-capped albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy. *Molecular Ecology*, **12**, 2747-2758.
- Baker GB, Gales R, Hamilton S, Wilkinson V (2002) Albatrosses and petrels in Australia: a review of their conservation and management. *Emu*, **102**, 71-97.
- Brothers N (1991) Albatross mortality and associated bait loss in the Japanese longline fishery in the Southern Ocean. *Biological Conservation*, **55**, 255-268.
- Brothers N, Gales R, Hedd A, Robertson G (1998) Foraging movements of the shy albatross *Diomedea cauta* breeding in Australia - implications for interactions with longline fisheries. *Ibis*, **140**, 446-457.
- Brothers NP, Reid TA, Gales RP (1997) At-sea distribution of shy albatrosses *Diomedea cauta cauta* derived from records of band recoveries and colour-marked birds. *Emu*, **97**, 231-239.
- Cornuet JM, Piry S, Luikart G, Estoup A, Solignac M (1999) New methods employing multilocus genotypes to select or exclude populations as origins of individuals. *Genetics*, **153**, 1989-2000.
- Croxall JP, Gales R (1998) An assessment of the conservation status of albatrosses. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 46-66. Surrey Beatty & Sons, Chipping Norton.
- Croxall JP, Prince PA, Rothery P, Wood AG (1998) Population changes in albatrosses at South Georgia. In: *Albatross: Biology and Conservation* (eds. Robertson G, Gales R), pp. 69-83. Surrey Beatty & Sons, Chipping Norton.

- Croxall JP, Wood AG (2002) The importance of the Patagonian Shelf for top predator species breeding at South Georgia. *Marine and Freshwater Ecosystems*, **12**, 101-118.
- Dalebout ML, Van Helden A, Van Waerebeek K, Baker CS (1998) Molecular genetic identification of southern hemisphere beaked whales (Cetacea, Ziphiidae). *Molecular Ecology*, **7**, 687-694.
- Double MC, Gales R, Reid T, Brothers N, Abbott CL (2003) Morphometric comparison of Australian shy and New Zealand white-capped albatrosses. *Emu*, **103**, 287-294.
- Eldridge MDB, Kinnear JE, Onus ML (2001) Source population of dispersing rock-wallabies (*Petrogale lateralis*) identified by assignment tests on multilocus genotypic data. *Molecular Ecology*, **10**, 2867-2876.
- Gales R (1993) Co-operative mechanisms for the conservation of albatross, p. 132. Australian Nature Conservation Agency and Australian Antarctic Foundation, Hobart, Tasmania.
- Gales R (1998) Albatross populations: status and threats. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 20-45. Surrey Beatty & Sons, Chipping Norton.
- Gales R, Brothers N, Reid T (1998) Seabird mortality in the Japanese tuna longline fishery around Australia, 1988-1995. *Biological Conservation*, **86**, 37-56.
- Greig C, Robertson JM, Banks MA (2002) Rapid PCR-based species tests for threatened sympatric salmonids. *Conservation Genetics*, **3**, 83-86.
- Griffiths R, Tiwari B (1995) Sex of the last wild spix's macaw. *Nature*, **375**, 454.
- Hansen MM, Ruzzante DE, Nielsen EE, Mensberg KLD (2000) Microsatellite and mitochondrial DNA polymorphism reveals life-history dependent interbreeding between hatchery and wild brown trout (*Salmo trutta* L.). *Molecular Ecology*, **9**, 583-594.
- Hedd A, Gales R (2001) The diet of shy albatrosses (*Thalassarche cauta*) at Albatross Island, Tasmania. *Journal of Zoology*, **253**, 69-90.

- Hedd A, Gales R, Brothers N (2001) Foraging strategies of shy albatross *Thalassarche cauta* breeding at Albatross Island, Tasmania, Australia. *Marine Ecology Progress Series*, **224**, 267-282.
- Hedd A, Gales R, Brothers N, Robertson G (1997) Diving behaviour of the shy albatross *Diomedea cauta* in Tasmania - initial findings and dive recorder assessment. *Ibis*, **139**, 452-460.
- Inchausti P, Weimerskirch H (2001) Risks of decline and extinction of the endangered Amsterdam albatross and the projected impact of long-line fisheries. *Biological Conservation*, **100**, 377-386.
- Klaer N, Polacheck T (1997) By-catch of albatrosses and other seabirds by Japanese longline fishing vessels in the Australian Fishing Zone from April 1992 to March 1995. *Emu*, **97**, 150-167.
- Manel S, Berthier P, Luikart G (2002) Detecting wildlife poaching: identifying the origin of individuals with Bayesian assignment tests and multilocus genotypes. *Conservation Biology*, **16**, 650-659.
- Murray TE, Bartle JA, Kalish SR, Taylor PR (1993) Incidental capture of seabirds by Japanese southern bluefin tuna longline vessels in New Zealand waters, 1988-1992. *Bird Conservation International*, **3**, 181-210.
- Nel DC, Ryan PG, Crawford RJM, Cooper J, Huyser OAW (2002a) Population trends of albatrosses and petrels at sub-Antarctic Marion Island. *Polar Biology*, **25**, 81-89.
- Nel DC, Ryan PG, Watkins BP (2002b) Seabird mortality in the Patagonian toothfish longline fishery around the Prince Edward Islands, 1996-2000. *Antarctic Science*, **14**, 151-161.
- Paetkau D, Calvert W, Stirling I, Strobeck C (1995) Microsatellite analysis of population structure in Canadian polar bears. *Molecular Ecology*, **4**, 347-354.
- Prince PA, Croxall JP, Trathan PN, Wood AG (1998) The pelagic distribution of South Georgia albatrosses and their relationships with fisheries. In: *Albatross:*

- Biology and Conservation* (eds. Robertson G, Gales R), pp. 137-167. Surrey Beatty & Sons, Chipping Norton.
- Prince PA, Rothery P, Croxall JP, Wood AG (1994) Population dynamics of black-browed and grey-headed albatrosses *Diomedea melanophris* and *D. chrysostoma* at Bird Island, South Georgia. *Ibis*, **136**, 50-71.
- Rannala B, Mountain JL (1997) Detecting immigration by using multilocus genotypes. *Proceedings of the National Academy of Sciences of the United States of America*, **94**, 9197-9201.
- Robertson CJR, Bell E, Schofield P (2003) Autopsy report for seabirds killed and returned from New Zealand fisheries, 1 October 2001 to 30 September 2002. Department of Conservation, Wellington, New Zealand.
- Ryan PG, Keith DG, Kroese M (2002) Seabird bycatch by tuna longline fisheries off southern Africa, 1998-2000. *South African Journal of Marine Science*, **24**, 103-110.
- Shivji M, Clarke S, Pank M, *et al.* (2002) Genetic identification of pelagic shark body parts for conservation and trade monitoring. *Conservation Biology*, **16**, 1036-1047.
- Tuck GN, Polacheck T, Bulman CM (2003) Spatio-temporal trends of longline fishing effort in the Southern Ocean and implications for seabird bycatch. *Biological Conservation*, **114**, 1-27.
- Weimerskirch H, Brothers N, Jouventin P (1997) Population dynamics of wandering albatross *Diomedea exulans* and Amsterdam albatross *D. amsterdamensis* in the Indian Ocean and their relationships with long-line fisheries - conservation implications. *Biological Conservation*, **79**, 257-270.



# Chapter IV



Copulation behaviour and paternity in  
shy albatrosses

## Abstract

Genetic data on the mating systems of several procellariiform seabirds have recently become available but behavioural data for these species are rarely obtained. I investigated the mating system of shy albatrosses (*Thalassarche cauta*) by combining genetic paternity analysis with observations during the pre-laying period. My main aims were to describe the copulatory behaviour of this species and identify how males achieve within-pair and extra-pair paternity. The vast majority of copulations occurred on the nest, were unforced, and were within-pair. Females controlled the success of copulations and were observed soliciting extra-pair matings. Within-pair and extra-pair copulations were behaviourally similar. A low frequency (7-10%) of extra-pair paternity was detected despite male use of frequent copulation as a paternity guard. The pre-laying foraging exodus of female shy albatrosses differed from that in other albatrosses: it was short in length and within-pair copulations occurred after the female's return two days prior to laying. This may reflect the close proximity of feeding grounds and the breeding colony.

## Introduction

Prior to the current ready availability of molecular methods of assessing paternity in vertebrate mating systems it was generally accepted that social monogamy, which was observed in the majority of bird species, corresponded to genetic monogamy. It has since been discovered however, that fewer than 25% of socially monogamous birds exhibit true genetic monogamy as defined by a total absence of extra-pair paternity (EPP; Griffith *et al.* 2002). Paternity thus cannot be inferred from observing social behaviour (Griffith *et al.* 2002).

Both males and females may benefit from extra-pair mating in a number of ways. For males, it may increase their fitness and their re-mating potential, and insure them against infertility in their social mate (Birkhead & Møller 1992). For females, it may allow them to gain better genes for their offspring, protect themselves against an infertile partner, acquire material benefits, and prevent inbreeding (Birkhead & Møller 1992). However, females that are long-lived and rely most heavily on paternal care of their offspring are expected to be maximally constrained in the extent to which they can enjoy these benefits without suffering losses of reproductive success caused by retaliatory desertion by their social mates (Mauck *et al.* 1999; Møller 2000). Therefore genetically monogamous mating systems remain a reasonable expectation for extremely long-lived taxa in which male parental care is vital, such as procellariiform seabirds. Intriguingly however, while 0% EPP has been confirmed in some Procellariiformes, including northern fulmars (*Fulmarus glacialis*; Hunter *et al.* 1992), Cory's shearwater (*Calonectris diomedea*; Swatschek *et al.* 1994), Leach's storm-petrel (*Oceanodroma leucorhoa*; Mauck *et al.* 1995), and Wilson's storm-petrel (*Oceanites oceanicus*; Quillfeldt *et al.* 2001), EPP has been detected in others, including: short-tailed shearwater (*Puffinus tenuirostris*; 9-13% EPP; Austin & Parkin 1996), waved albatross (*Phoebastria irrorata*; 25% EPP; Huyvaert *et al.* 2000), black-browed albatross (*Thalassarche melanophrys*; up to 8%

EPP; Burg 2000), grey-headed albatross (*Thalassarche chrysostoma*; up to 10% EPP; Burg 2000), and wandering albatross (*Diomedea exulans*; up to 17% EPP; Burg 2000).

Genetic studies of avian mating systems are most informative when interpreted in the context of complementary behavioural data (Gowaty & Gibbs 1993). However, data on the reproductive behaviour of procellariiform seabirds are notably lacking, and where available are inconsistent with genetic data (Hunter *et al.* 1992). Many species are nocturnal, breed in underground burrows, and inhabit remote oceanic islands, which makes them difficult to observe. These hindrances are absent in shy albatrosses (*Thalassarche cauta*). Shy albatrosses are long-lived, socially monogamous seabirds that form long-term pair bonds and breed in densely populated colonies on three islands near Tasmania, Australia. Females lay a single egg each year and biparental care is obligatory, with both sexes heavily involved in nest defence, incubation, and chick provisioning. Very little is known about their reproductive behaviour and within-pair fidelity.

I investigated the mating system of shy albatrosses by combining pre-laying observations with genetic paternity analysis. My main aims were to (1) describe the copulatory behaviour of shy albatrosses; (2) elucidate the genetics of their mating system; and (3) identify how males achieve within-pair and extra-pair paternity.

## Methods

This study investigates the mating system of shy albatrosses breeding at Albatross Island (40°23'S, 144°39'E), Tasmania, Australia, which hosts c. 5000 breeding pairs (Brothers *et al.* 1998).

## Observational data

Most shy albatross eggs are laid in September (Gales 1998). I observed pre-laying/laying behaviours in a study plot of 48 nests from 7 to 21 September 2001, and 12 to 21 September 2002. One or two observers made observations from a canvas hide that provided an unobstructed view of all study nests. Dawn and dusk are periods of high activity in shy albatross colonies (Rosemary Gales, pers. comm.) hence the majority of observational data were collected during these times. Of the 15 days of data collection in 2001, watches were conducted for 7 h 45 min each day (0600-0900, 1100-1300, and 1600-1845 AEST) on nine days, for 5 h 45 min each day (0600-0900 and 1600-1845) on five days, and for 3 h (0600-0900) on one day. Of the 10 days of data collection in 2002, watches were conducted for 6 h each day (0545-0845 and 1600-1900) on seven days, for 3 h each day (0545-0845) on two days, and for 4 h 30 min (0815-1000 and 1600-1845) on one day. Most (94/96) breeding birds within the site were marked with a unique combination of two colour bands during incubation in 2001. Therefore observations made prior to laying in 2001 were on unmarked birds, whereas those in 2002 were on marked birds. The sex of individual study birds was determined during copulation.

The location, duration, success, number of cloacal contacts, female receptivity, and, where possible, whether it was within- or extra-pair were recorded for each copulation attempt observed, as well as whether or not it was forced. Copulation attempts were considered forced if the female behaved aggressively towards the male while he mounted or attempted to mount her.

Colony attendance patterns were investigated by timing the arrivals and departures of breeding birds. In addition, whether the nest was left unattended or was attended by one or both members of the breeding pair was recorded at 11 times throughout each day in 2001.



The exact date of egg laying was recorded for most study nests, the exceptions being eggs that were laid before my arrival to, or after my departure from, the island each year. All means are reported  $\pm$  standard deviation (SD).

### **Genotyping and parentage analysis**

The genetic mating system of shy albatrosses was investigated by blood sampling adults and chicks within the study site and assessing parentage using microsatellite DNA markers. Adults were sampled during the non-breeding period (June/July) and chicks were sampled at their nests either shortly after hatching in December or just prior to fledging in April. Sampling a sufficient number of adults was difficult because individuals are more flighty during the non-breeding period and are therefore harder to capture. Thus as I was interested in investigating paternity in shy albatrosses I prioritised sampling the breeding male at each nest and only sampled females opportunistically.

Over the two breeding seasons the breeding male and a total of 29 chicks were sampled at 21 nests: the data for 8 nests being obtained in successive seasons. These individuals were genotyped at an average of 7.25 ( $\pm$  0.8) polymorphic microsatelliteloci, five to six of which were autosomal and up to three of which were Z-linked. As males are the homogametic sex in birds, the paternal inheritance of Z-linked markers is identical to that of autosomal ones: every offspring carries one paternally inherited Z-linked allele. Details on the isolation, PCR primers, amplification conditions, and descriptive statistics for autosomal loci are described in Abbott & Double (2003 [Chapter II]). Information on the Z-linked loci is available on request.

To investigate paternity, autosomal genotypic data were used to identify a group of candidate genetic fathers for each chick, which comprised all males without any

genetic mismatches, using CERVUS 2.0 (Marshall *et al.* 1998). Z-linked loci were not included in this step because the software assumes Hardy-Weinberg equilibrium, which is violated in sex-linked loci due to an excess of homozygotes. Then a conservative approach was taken to select the most likely father from this subset. If the male member of the breeding pair at the sampled nest was in the group of candidate fathers, and had no mismatches at Z-linked loci, paternity was assigned to him. If the social father was not selected as a candidate father, the genotypic data, including Z-linked loci, were checked manually to confirm the presence and accuracy of genetic mismatches before this male was excluded. Exclusion probabilities based on allele frequencies of autosomal loci were calculated using CERVUS 2.0 (Marshall *et al.* 1998).

Over the two seasons I sampled seven breeding females at their nests and a total of 10 chicks: two chicks across the two seasons at three nests, and one chick across the two seasons at four nests. Autosomal genotypes of each mother-chick pair were checked manually for any evidence of mismatches.

## Results

Observational data were collected for a total of 101 h in 2001 and 50 h in 2002, which amounted to daily averages of 6 h 45 min and 5 h in each year, respectively. A total of 724 copulation attempts, defined as all acts whereby one bird (presumed male) mounted or attempted to mount another bird (presumed female), were observed. Egg laying dates were recorded for 42 study nests in 2001 and 25 nests in 2002.

## Copulation behaviour

Prior to copulations the female became seated on the nest if she was not already in this position. The male would then mount her and orient himself so that he was facing the same direction before lowering his body to rest against her back. Next, the male would lower his head and repeatedly tap his bill against the female's bill, while at the same time extending his tail out behind him and swinging it rapidly from side to side. During this time the female would deliberately raise her tail so that it came into contact with the male's tail, allowing him to eventually push her tail towards one side while bending his tail downwards to bring his cloaca into contact with hers.

Both birds would remain still for a few seconds while cloacal contact occurred and presumably sperm was being transferred. Following cloacal contact the female kept her tail raised and contracted her cloaca repeatedly, and the male usually dismounted, although in rare instances he rested on top of the female for several minutes.

Nest sites were rarely left unattended; one member of the breeding pair typically stayed on the nest and defended it against neighbours and passing birds. Before egg laying, males attending their nests were regularly seen displaying to passing females when their own mate was absent. Once the egg was laid and incubation began this behaviour ceased. Passing females tended to move through the colony slowly and extend their beaks towards individuals attending their nests (similar to 'pointing' behaviour described by Pickering & Berrow 2001).

Observed copulations occurred within the colony perimeter, despite that birds were commonly seen congregating in regions bordering the colony that were easily visible from the hide. The large majority of copulation attempts were unforced (96%,  $n = 724$ ) and occurred on the nest (96%,  $n = 724$ ), with the remainder occurring immediately beside the nest. Females controlled the success of copulations through their tail position.



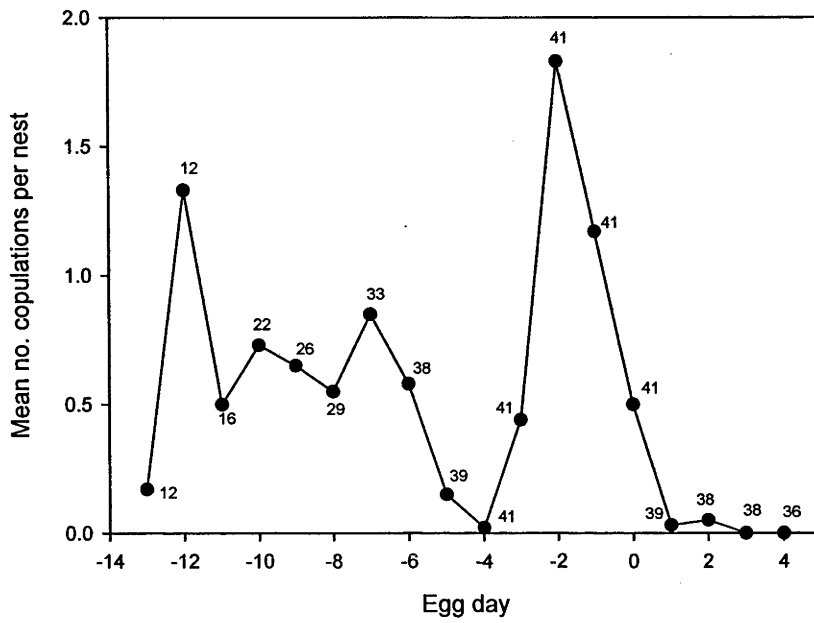
### **Within-pair copulations**

A total of 689 within-pair copulation attempts were observed. Their mean duration, measured as the length of time the male spent mounted on the female, was 2 min 02 s ( $\pm 1.00$  min) for successful copulations and 1 min 31 s ( $\pm 2.00$  min) for unsuccessful copulations. Females were usually receptive to their mate: the female raised her tail in 76% of all within-pair copulation attempts, 72% of which were successful, resulting in one to six cloacal contacts (mean  $1.7 \pm 1.0$ ) per mount. The female kept her tail lowered and was hence responsible for most (85%,  $n = 191$ ) failed copulation attempts, however in 160 (84%) of these the same pair was observed copulating successfully at some other time during the same day. Hence, most failed within-pair copulation attempts appeared to reflect factors such as poor male positioning or the female's disinterest in copulating repeatedly over a short time frame, as opposed to signalling low female receptivity. Remarkably, one copulation was observed while an egg was protruding from the female's cloaca.

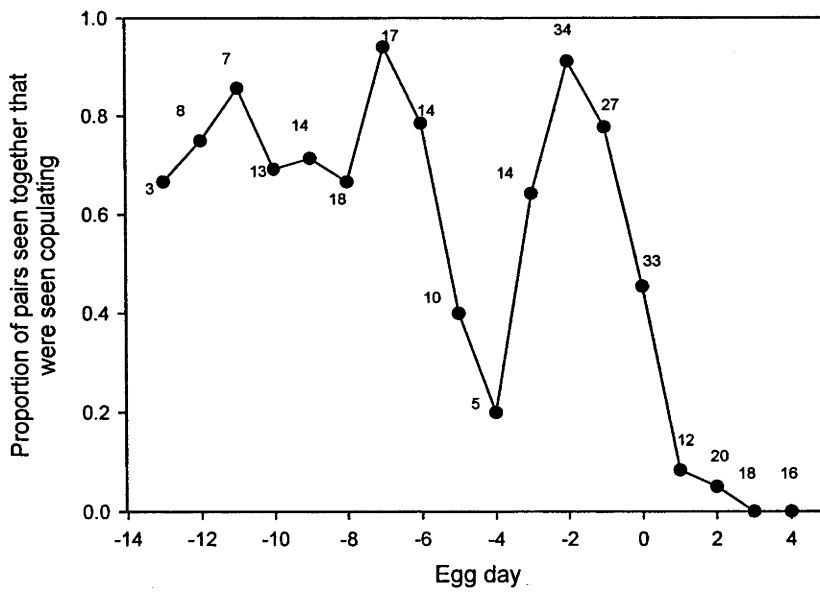
### **Frequency of within-pair copulations and colony attendance patterns**

Within-pair copulations occurred regularly prior to egg laying and ceased almost immediately after the egg was laid (Figure 1A). Across 12 nests observed from days -13 through -1 (with egg laying on day 0) in 2001, a mean of  $10.3 (\pm 3.3)$  copulations per nest were observed. The proportion of pairs seen together at their nest that was also seen copulating successfully during egg days -13 to 4 is shown in Figure 1B, and indicates that prior to egg laying, most pairs (mean  $69\% \pm 20\%$ ) copulated if they were both present at the nest. Pre-laying copulations were commonly observed following the reunion of a breeding pair at their nest after the arrival of the member that had been absent (presumably at sea). Again, I investigated this using data on nest arrivals at 12 nests observed in 2001 from days -13 through -1. After excluding two arrivals that occurred within 5 min of the end of a watch period, I found that a

(A) Overall copulation rate

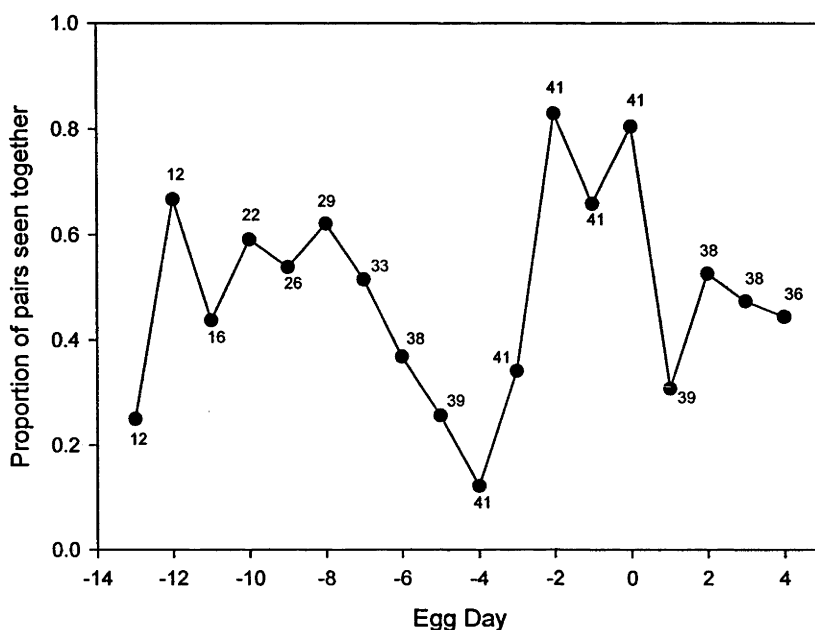


(B) Frequent within-pair copulations



(Continued next page)

(C) Pre-laying exodus



**Figure 1.** (A) Mean number of successful within-pair copulations observed per nest on egg days -13 through 4. Numbers beside each data point indicate the total number of study nests observed on each day. (B) The proportion of Shy Albatross pairs seen together at their nest that was also seen copulating on egg days -13 through 4. Numbers beside each data point indicate the number of nests at which a pair was seen together on each day. (C) The proportion of Shy Albatross pairs seen together at their nest on egg days -13 through 4. Numbers beside each data point indicate the total number of study nests observed on each day.

successful copulation followed 24 out of 32 (75%) nest arrivals, and occurred an average of 26 min 42 s ( $\pm$  30 min 35 s) after the arrival.

Females were generally absent from the colony between egg days -5 and -3, which corresponded to a severe drop in pair attendance and within-pair copulation frequency (Figures 1C and 1B). Females almost invariably returned to the nest on day -2 and remained until after egg laying, which resulted in a peak in pair attendance between days -2 and 0 (Figure 1C). Most (88%) pairs were seen copulating in the two days prior to egg laying, with a peak in copulation frequency on day -2 (Figures 1A and 1B).

### **Extra-pair copulatory behaviour**

A total of nine extra-pair copulations were observed; six in 2001 and three in 2002. Eight of these involved an extra-pair female of unknown identity visiting a male at his nest while his partner was absent. One male was visited by an unbanded extra-pair female early in the morning two days in a row and a successful copulation resulted both times, though whether this was the same female both days was unknown. In one instance a female that had been rejected by her paired male shortly before egg laying (described below) copulated with the male at an adjacent nest. All extra-pair copulations occurred on the male's nest and were unforced. On average, extra-pair copulations lasted 2 min 15 s ( $\pm$  1 min 37 s) and resulted in 2.1 ( $\pm$  1.2) cloacal contacts, and hence did not differ greatly from within-pair copulations. However, while paired birds were often together at the nest for extended periods of time, females departed shortly after extra-pair copulations.

### Forced copulation attempts

Only 26 of 724 (3.6%) observed copulation attempts were forced, nine of which occurred in 2002 when birds were colour banded. In each case a breeding male attending his nest was involved. The male mounted or attempted to mount either a breeding female attending a nearby nest (7/9) or an unidentified bird that was moving past his nest (2/9). The male successfully mounted the female in only 42% ( $n = 26$ ) of forced copulation attempts, and in only one case did the female ultimately raise her tail to allow for a successful copulation. This occurred at a nest site that was not well established; although a male defended the site, a female was only rarely seen there and a nest bowl had not been made. The female involved in this copulation was not a known study bird. Forced mount attempts usually lasted only a few seconds, and appeared to be acts of territorial aggression, rather than legitimate attempts at copulation. Huyvaert *et al.* (2000) made a similar suggestion for waved albatrosses. Similar to unforced copulations that did not fully cease until shortly after egg laying (Figure 1B), a combined total of five forced copulation attempts occurred on egg days 0 and 1.

### Divorce

A male-mediated divorce was observed in 2002 at a study nest with an established pair (they had an unsuccessful breeding attempt in 2001). When the paired female reunited with her mate at the nest on egg day -2, he acted aggressively towards her. She then copulated with a paired male at an adjacent nest just six minutes after her initial arrival. For the next two days she made further unsuccessful attempts to approach her mate, and then laid her egg at a vacant site that she soon abandoned.

### Parentage assignment

The combined exclusion probability for the six autosomal loci used for parentage analysis was 91% when the maternal genotype was known, and 73% when it was unknown, the latter being the case for most sampled chicks (22/29). These figures may reflect relatively low allelic diversity (mean of four alleles per locus, see Abbott & Double 2003 [Chapter II]) or genetic clustering of related individuals in the study site. Regardless, a mean of 5.0 ( $\pm 2.1$ ) candidate fathers genetically matched each sampled chick ( $n = 29$ ). No genetic mismatches were found in any genotypic comparison of mother-chick ( $n = 10$ ) at five to six autosomal loci, hence there was no evidence of nest parasitism or mutation.

At 26 of 29 (90%) study nests, the social father and the sampled chick matched at each locus and so these chicks were classified as within-pair. The remaining three chicks had genetic mismatches with their social father. The first of these chicks mismatched its social father at one of six autosomal loci and one of two Z-linked loci. The second chick mismatched its social father at two of five autosomal loci and one of three Z-linked loci. The third chick mismatched its social father at one of six autosomal loci (Z-linked data were not available). Depending on whether one mismatch is taken as sufficient evidence to exclude a social father, the rate of EPP in this study was between 6.9% (2/29) and 10.3% (3/29). This range is a conservative estimate since social fathers that had no genetic mismatches with their chick were automatically assigned paternity over other candidate fathers that also genetically matched the chick.

### Discussion

My observations of the copulatory behaviour of shy albatrosses revealed that the vast majority of copulations occurred on the nest, were unforced, and were within-pair.

Females controlled the success of all copulations and in a small number of instances were observed soliciting extra-pair matings. Extra-pair copulations were behaviourally similar to within-pair copulations, and resulted in a low frequency of EPP.

### Timing of the fertile period

The timing of the fertile period in shy albatrosses is difficult to assess. In albatrosses, there is a delay in ovulation after yolk formation (Astheimer *et al.* 1985) that coincides with a pre-laying foraging exodus that is common in Procellariiformes (Astheimer & Grau 1990). The exodus is thought to allow females to supplement their nutrient reserves as needed for egg production in the days immediately prior to laying (Astheimer & Grau 1990). Excluding single-day visits, female black-browed and grey-headed albatrosses are absent from their colonies for an average of 10 and 16 days respectively (Astheimer *et al.* 1985). I found the pre-laying exodus of female shy albatrosses to be comparatively short, lasting only a few days, which may be because breeding shy albatrosses are relatively sedentary and rely heavily on local food resources (Brothers *et al.* 1998; Hedd *et al.* 2001).

Several studies provide evidence of sperm storage in female Procellariiformes (Astheimer *et al.* 1985; Austin & Parkin 1996; Hunter *et al.* 1992). In black-browed and grey-headed albatrosses, most pairs do not copulate after the female leaves on her exodus, and she lays two days after her return to the colony (Astheimer *et al.* 1985). The last copulation and egg laying are also temporally separated in northern fulmars and short-tailed shearwaters, by an average of 16 and 21 days respectively (Austin & Parkin 1996; Hunter *et al.* 1992). Hence in these species, the fertile period effectively ends once the female leaves on her exodus regardless of the timing of fertilisation (Hunter 1998). In shy albatrosses however, a peak in copulation rate occurs upon the female's return from her pre-laying absence. Since fertilisation in

albatrosses is thought to occur within the few days prior to laying (Astheimer *et al.* 1985), it is possible that shy albatross eggs are fertilised by sperm inseminated during this time. Alternatively, the peak in copulation rate on day -2 may reflect the general tendency toward frequent within-pair copulations, and in particular the tendency of pairs to copulate after being reunited at the nest after a period of separation.

### **Frequent copulations**

While a very small number of copulations are required for successful fertilisation, many colonially breeding birds copulate frequently (Birkhead & Møller 1992). It is thought that frequent copulation is the best paternity guard if ecological constraints prevent direct mate-guarding, which occurs when males follow their mates closely while they are fertile (Birkhead & Møller 1992; Møller & Birkhead 1991). The only published study on the mating behaviour of a procellariiform seabird found that northern fulmars successfully use frequent copulation to assure paternity (Hunter *et al.* 1992). Shy albatross breeding pairs copulate regularly and repeatedly prior to egg laying, and mate-guarding was not observed, so shy albatrosses also seem to use this strategy.

Both sexes are likely to benefit from frequent within-pair copulations. For males, the probability of fertilisation increases with the amount of sperm he inseminates, and if he is the last male to copulate with a female before her eggs are fertilised (Birkhead & Møller 1992). In northern fulmars, pair males were always the last male to copulate with their mates, and they always gained paternity despite females engaging in extra-pair matings (Hunter *et al.* 1992). A similar strategy of pair males trying to gain last sperm advantage may be important for shy albatrosses, which is consistent with the large fraction of pairs that copulated once reunited after one member had been absent. Female shy albatrosses may benefit from copulating frequently with



their social mate for reasons other than ensuring his paternity. If males assess their likelihood of paternity through the female's willingness to copulate with him, and may opt to divorce her or to refrain from investing care if his confidence of paternity is low, it will be in the best interest of females to copulate frequently with their mates (Westneat & Stewart 2003). The male-mediated divorce seen in this study confirms that male shy albatrosses do indeed reject their social mate in some circumstances, which were not discernible in the observed case.

### **Extra-pair paternity**

My finding of 7-10% EPP in shy albatrosses is higher than the average frequency of 3% EPP found among 23 non-passerines (Westneat & Sherman 1997), but is within the range reported for other albatross species. Burg (2000) found 0-8% EPP in black-browed albatrosses, 2-10% EPP in grey-headed albatrosses, and 6-17% EPP in wandering albatrosses. A much higher 25% EPP was detected in waved albatrosses (Huyvaert *et al.* 2000). Behavioural data are not available for these species, however the possibility that EPP in waved albatrosses is at least partially attributable to forced extra-pair copulations has been suggested (Huyvaert *et al.* 2000). I found no evidence that forced copulations are part of the mating system of shy albatrosses: forced mounts were rare and did not appear to lead to successful sperm transfer.

Extra-pair copulations observed in shy albatrosses resulted from females travelling through the colony; females visited extra-pair males at their nests, which is where extra-pair copulations occurred. Evidence of female pursuit of extra-pair copulations has been found together with genetic evidence of EPP in only 12 other species (Westneat & Stewart 2003). In northern fulmars, females solicited 14% of observed extra-pair copulations, but no EPP occurred (Hunter *et al.* 1992).

Detection of EPP in shy albatrosses is intriguing given that many factors are predicted to restrict it. First, although it is impossible to identify conclusively the potential benefits to female shy albatross of seeking extra-pair copulations (see Griffith *et al.* 2002), several possibilities appear unlikely based on results presented here. Female shy albatrosses do not receive material resources from engaging in extra-pair matings, as occurs in Adelie penguins (*Pygoscelis adeliae*; Hunter & Davis 1998). Extra-pair mating for infertility insurance is not expected in such a long-lived species, since individuals could assess the outcome of early matings and divorce if no young hatched. Further, two out of the three extra-pair shy albatross chicks identified here occurred at nests that fledged a within-pair chick in the other year studied. Mate fidelity between reproductive seasons is very high (>90%) in albatrosses for which data are available (Bried & Jouventin 2002), making it unlikely that females use extra-pair copulations to seek out a better mate in future years. Further, frequent within-pair copulations in shy albatrosses (as discussed above) presumably lower the chance of extra-pair males gaining paternity.

Second, currently popular models for explaining observed EPP rates in terms of the risks of retaliation incurred by unfaithful females (see Arnold & Owens 2002) would predict that the life history characteristics of shy albatrosses strongly favour genetic monogamy. Expectations include that males of species with short reproductive lives should refrain from abandoning a brood with higher levels of EPP than males of species with long reproductive futures, for whom 'each breeding event represents a smaller proportion of potential lifetime reproductive success' (Mauck *et al.* 1999:99). It is also predicted that high survival of both males and females in long-lived species leads to a high likelihood of future mating opportunities for social pairs, thereby subjecting females to a real threat of divorce if they cuckold their mate (Arnold & Owens 2002). Indeed, using a dynamic programming approach Mauck *et al.* (1999) found that variation in adult mortality rates accounts for 50% of the variation in EPP rates. A second prediction of the risk of retaliation hypothesis is a negative

association between EPP rates and the necessity of parental care for offspring survival (Arnold & Owens 2002; Griffith *et al.* 2002). The expectation is that the degree to which female reproductive success is dependent on male parental care will be reflected in the intensity of selection pressure favouring mate-retention strategies in females. Results of phylogeny-based comparative analyses undertaken by Arnold & Owens (2002) and Møller (2000) both support this prediction, with high EPP rates associated with reduced need for parental care. Therefore as long-lived taxa with obligate reliance on biparental care, unfaithful female shy albatrosses are expected to be especially vulnerable to losses in reproductive success as a result of retaliatory behaviour of her mate.

In light of these factors predicted to limit EPP in shy albatrosses, perhaps it is surprising that their rate of EPP approaches the average of 11% EPP among socially monogamous bird species (Griffith *et al.* 2002). However, ecological factors may promote EPP in shy albatrosses. The preclusion of mate guarding by both long foraging trips and the need for constant nest site defence, in combination with the ready availability of extra-pair mates in densely-populated breeding colonies, are together predicted to generate ample opportunities for extra-pair copulations (Birkhead *et al.* 1987). Indeed, higher extra-pair copulation rates have been found among colonial bird species than among solitary breeders (Birkhead & Møller 1992). Further, while many potential benefits to female shy albatrosses from engaging in extra-pair matings have been excluded based on results of this study (as discussed above), in light of their high mate fidelity between breeding seasons it remains possible that they seek extra-pair sires to increase genetic diversity among their offspring.

## References

- Abbott CL, Double MC (2003) Genetic structure, conservation genetics, and evidence of speciation by range expansion in shy and white-capped albatrosses. *Molecular Ecology*, **12**, 2953-2962.
- Arnold KE, Owens IPF (2002) Extra-pair paternity and egg dumping in birds: life history, parental care and the risk of retaliation. *Proceedings of the Royal Society of London Series B-Biological Sciences*, **269**, 1263-1269.
- Astheimer LB, Grau CR (1990) A comparison of yolk growth rates in seabird eggs. *Ibis*, **132**, 380-394.
- Astheimer LB, Prince PA, Grau CR (1985) Egg formation and the pre-laying period of black-browed and grey-headed albatrosses *Diomedea melanophris* and *D. chrysostoma* at Bird Island, South Georgia. *Ibis*, **127**, 523-529.
- Austin JJ, Parkin DT (1996) Low frequency of extra-pair paternity in two colonies of the socially monogamous short-tailed shearwater *Puffinus tenuirostris*. *Molecular Ecology*, **5**, 145-150.
- Birkhead TR, Atkin L, Møller AP (1987) Copulation behavior of birds. *Behaviour*, **101**, 101-138.
- Birkhead TR, Møller AP (1992) *Sperm Competition in Birds*. Academic Press, London.
- Bried J, Jouventin P (2002) Site and mate choice in seabirds: an evolutionary approach. In: *Biology of Marine Birds* (eds. Schreiber EA, Burger J). CRC Press, Boca Raton, USA.
- Brothers N, Gales R, Hedd A, Robertson G (1998) Foraging movements of the shy albatross *Diomedea cauta* breeding in Australia - implications for interactions with longline fisheries. *Ibis*, **140**, 446-457.
- Burg T (2000) *Genetic Analyses of Albatrosses: Mating Systems, Population Structure and Taxonomy*. PhD, Cambridge University, UK.

- Gales R (1998) Albatross populations: status and threats. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 20-45. Surrey Beatty & Sons, Chipping Norton.
- Gowaty PA, Gibbs HL (1993) DNA fingerprinting in avian behavioral ecology: two cultures arise. *Auk*, **110**, 152-155.
- Griffith SC, Owens IPF, Thuman KA (2002) Extra pair paternity in birds: a review of interspecific variation and adaptive function. *Molecular Ecology*, **11**, 2195-2212.
- Hedd A, Gales R, Brothers N (2001) Foraging strategies of shy albatross *Thalassarche cauta* breeding at Albatross Island, Tasmania, Australia. *Marine Ecology Progress Series*, **224**, 267-282.
- Hunter FM (1998) Timing of pair and extra-pair copulations in the northern fulmar. *Condor*, **100**, 141-145.
- Hunter FM, Burke T, Watts SE (1992) Frequent copulation as a method of paternity assurance in the northern fulmar. *Animal Behaviour*, **44**, 149-156.
- Hunter FM, Davis LS (1998) Female Adelie penguins acquire nest material from extrapair males after engaging in extrapair copulations. *Auk*, **115**, 526-528.
- Huyvaert KP, Anderson DJ, Jones TC, Duan WR, Parker PG (2000) Extra-pair paternity in waved albatrosses. *Molecular Ecology*, **9**, 1415-1419.
- Marshall TC, Slate J, Kruuk LEB, Pemberton JM (1998) Statistical confidence for likelihood-based paternity inference in natural populations. *Molecular Ecology*, **7**, 639-655.
- Mauck RA, Marschall EA, Parker PG (1999) Adult survival and imperfect assessment of parentage: effects on male parenting decisions. *The American Naturalist*, **154**, 99-109.
- Mauck RA, Waite TA, Parker PG (1995) Monogamy in Leach's storm-petrel: DNA-fingerprinting evidence. *The Auk*, **112**, 473-482.
- Møller AP (2000) Male parental care, female reproductive success, and extrapair paternity. *Behavioral Ecology*, **11**, 161-168.

- Møller AP, Birkhead TR (1991) Frequent copulations and mate guarding as alternative paternity guards in birds: a comparative study. *Behaviour*, **118**, 170-186.
- Pickering SPC, Berrow SD (2001) Courtship behaviour of the wandering albatross *Diomedea exulans* at Bird Island, South Georgia. *Marine Ornithology*, **29**, 29-37.
- Quillfeldt P, Schmoll T, Peter HU, Epplen JT, Lubjuhn T (2001) Genetic monogamy in Wilson's storm-petrel. *Auk*, **118**, 242-248.
- Swatschek I, Ristow D, Wink M (1994) Mate fidelity and parentage in Cory's shearwater *Calonectris diomedea* - field studies and DNA fingerprinting. *Molecular Ecology*, **3**, 259-262.
- Westneat DF, Sherman PW (1997) Density and extra-pair fertilizations in birds: a comparative analysis. *Behavioral Ecology and Sociobiology*, **41**, 205-215.
- Westneat DF, Stewart IRK (2003) Extra-pair paternity in birds: causes, correlates, and conflict. *Annual Review of Ecology and Systematics*, **34**, 365-396.



## Chapter V



Duplicate mitochondrial control regions  
in *Thalassarche* albatrosses consist of  
both independently and concertedly  
evolving segments

## Abstract

This study characterizes a novel mitochondrial DNA duplication near and including the control region in *Thalassarche* albatrosses using direct DNA sequencing and restriction digest mapping. The duplicate control regions align easily, are not identical in sequence or in length, and do not contain evidence of degeneration. Phylogenetic analyses of domain I of both control region copies in five *Thalassarche* species indicate that they are largely evolving in concert, however a short section within them is clearly evolving independently. To my knowledge this is the first time contrasting evolutionary patterns have been reported for duplicate control regions. Gene conversion is considered a possible mechanism for the maintenance of concertedly evolving duplicate control regions found in other taxa, and may be a plausible mechanism behind the complex evolutionary patterns described here. Available evidence suggests that this duplication may be taxonomically widespread, probably across all Procellariiformes and could potentially occur in other closely related avian groups.



## Introduction

The control region is a noncoding mitochondrial DNA (mtDNA) sequence involved in the regulation of replication and transcription of the mitochondrial genome (Ruokonen & Kvist 2002). It has proven to be a valuable tool in phylogenetic, phylogeographic, and population genetic studies, due in part to its rapid rate of evolution, haploidy, and maternal inheritance (Sorenson & Quinn 1998). However, recent investigations have revealed important caveats associated with using the control region in evolutionary studies, including transposition of mtDNA to the nuclear genome (see Zhang & Hewitt 1996), which can confound the acquisition of authentic mtDNA sequence data. In addition, gene rearrangements and duplications involving the control region have recently been discovered in a wide variety of taxa, including parrots (Eberhard *et al.* 2001), metastriate ticks (Black & Roehrdanz 1998; Campbell & Barker 1999), sea cucumbers (Arndt & Smith 1998), snakes (Kumazawa *et al.* 1996), and self-fertilizing fish (Lee *et al.* 2001). Although it was initially thought that selection for small genome size in mtDNA would result in duplicated segments having a relatively short lifespan (Moritz & Brown 1987), recent studies have revealed control region duplications which have persisted through multiple speciation events and in which the functionality of both copies appears to have been maintained (Arndt & Smith 1998; Black & Roehrdanz 1998; Campbell & Barker 1999; Eberhard *et al.* 2001; Kumazawa *et al.* 1996). Clearly, an understanding of how duplicate control regions evolve is needed if one or both regions are being targeted for deciphering evolutionary relationships.

In this study I describe an alteration in mtDNA gene organisation in albatross species of the genus *Thalassarche* that involves a control region duplication initially detected in an evolutionary study (Abbott & Double 2003b [Chapter I]). The gene order found here has not been previously described. The two control region copies were evaluated for evidence of degeneration by comparing them with conserved sequence

blocks of functional significance that are ubiquitous to animal control regions. Duplicate control regions within individuals of five *Thalassarche* species examined contained sequence variation in domain I, therefore this region was targeted for phylogenetic analyses to investigate patterns of evolution in the two control regions. My results did not identify either control region copy as being degenerate, and indicate that although they are evolving predominantly in concert, a small section within them is clearly evolving independently. To my knowledge this is the first time contrasting evolutionary patterns have been found within duplicated control regions. Available evidence suggests that this duplication may be taxonomically widespread, so the results presented here should be considered in future evolutionary studies targeting the control region of all Procellariiformes and potentially other closely-related avian groups.

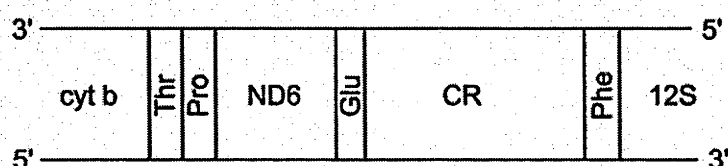
## Methods

This study was prompted by the discovery of sequence heteroplasmy in domain I of the mitochondrial control region of *Thalassarche* albatrosses during a phylogeographic study (Abbott & Double 2003b [Chapter I]). At that time, suitable polymerase chain reaction (PCR) primers for the control region of these taxa were not available, hence they were developed *de novo* by first amplifying the region between the genes for cytochrome *b* (*cytb*) and 12S as it typically contains the control region. This was done by ‘long-range’ PCR using primers cbF1 and 12sR1 (Table 1, Figure 1) as described in Abbott & Double (2003b [Chapter I]). A PCR product thought to be approximately 3000 base pairs (bp) in length was obtained and subsequently sequenced by repeatedly designing internal primers, which are shown in Table 1 and Figure 1. Ultimately I obtained sequence for *T. cauta* from *cytb* to 12S that appeared to represent the typical avian gene order (Figure 1; Desjardins & Morais 1990), although forward and reverse sequences in domain III of the control

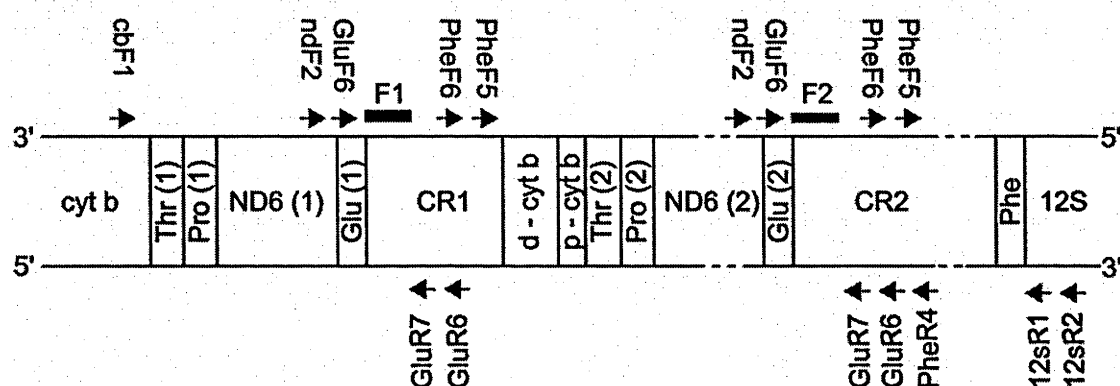
**Table 1.** mt DNA PCR primers used in this study. Primers with two priming sites due to the duplication (see Figure 1) are marked with an asterisks.

Primer	Location	Sequence 5' to 3'	Source
cbF1	cytb	ATGAATCGGCAGCCAACCAAGTAG	Abbott & Double 2003b [Chapter I]
ndF2	ND6*	GATCTGCTGCCAAAGATACAGAG	This study
GluF6	ND6*	AGGATTAGACGCAACTGCCAGC	Abbott & Double 2003b [Chapter I]
PheF6	CR*	CCTTTTTTTTTTGGGGCGTC	This study
PheF5	CR*	TTTCACTTGTCATCAACACTGG	This study
GluR7	CR*	CGGGTTGCTGATTTCCTCG	Abbott & Double 2003b [Chapter I]
GluR6	CR*	GACGCCCCAAAAAAGG	Abbott & Double 2003b [Chapter I]
PheR4	CR	TTGTTTTGGCAGGGAGGCTC	This study
12sR2	12S	GCTATAACTAAGTCAAGCTTTCGC	This study
12sR1	12S	TAAGCGTTTGTGCTCGTAGTTCTC	Abbott & Double 2003b [Chapter I]

(a) Standard avian cytochrome *b* to 12S gene order



(b) *T. cauta* cytochrome *b* to 12S gene order



**Figure 1.** Schematic comparison (not drawn to scale) of the *cytb* to 12S mitochondrial gene order found in most birds (a) with that reported here in *T. cauta* (b). Approximate locations of PCR primers used in this study are shown by arrows (note that some primers have two priming sites due to duplicated sequence). tRNA genes are labelled using the abbreviation of their associated amino acid and multiple copies of identically duplicated regions are distinguished by numbers given in parentheses after the name. The locations of cloned fragments from each control region used for phylogenetic analyses are labelled as F1 and F2 and are indicated by solid black boxes. Regions for which direct sequence data were not obtained are indicated by dotted horizontal margins rather than solid lines.

region could not be aligned with confidence due to the presence of several hundred base-pairs of complex repeats (Lunt *et al.* 1998).

Initial amplifications of domain I of the control region of *T. cauta* used primers GluF6 and GluR6 (Table 1, Figure 1) and yielded ambiguous sequence characterized by double peaks at some sites. PCR products were then cloned to obtain unambiguous sequences, which uncovered two unique sequences, 327 bp in length, within a single individual, hereafter referred to as F1 and F2 fragments (Figure 1). PCR primers were designed to specifically amplify each of these fragments. Detailed methods for these amplifications and cloning procedures are described by Abbott & Double (2003b [Chapter I]).

From this point I endeavoured to identify the source of this apparent sequence heteroplasmy and investigated three possible hypotheses: (1) co-amplification of a nuclear homolog; (2) a mtDNA duplication involving the control region; and (3) ‘true’ heteroplasmy caused by the co-existence of multiple non-identical mtDNA molecules within individuals.

Nuclear copies of the control region have been found in other bird species (Kidd & Friesen 1998; Ruokonen *et al.* 2000; Sorenson & Fleischer 1996). To evaluate the hypothesis that either F1 or F2 was nuclear in origin, the inheritance pattern of each was investigated by sequencing families of *T. cauta* sampled at Albatross Island, Tasmania, Australia. Seven mother/father/chick trios, and three mother/chick pairs were examined. Each chick was confirmed to be the genetic progeny of the sampled parents using five to six variable nuclear microsatellite markers (see Abbott & Double 2003a [Chapter II] for amplification conditions and descriptive statistics of each marker).

From the study of inheritance it was hypothesized that the F1 and F2 sequences were both mitochondrial (see Results and Discussion). It was therefore necessary to distinguish between the possibilities that they were located within the same mtDNA molecule, which would suggest a duplication event, or that two different mtDNA molecules coexisted within individuals (ie. mitochondrial heteroplasmy). mtDNA heteroplasmy is generally thought to be exceptionally rare, and although it has been argued otherwise (Grzybowski 2000), evidence for its occurrence is scarce. In contrast, several recent reports of control region duplications have emerged (Arndt & Smith 1998; Bensch & Harlid 2000; Black & Roehrdanz 1998; Campbell & Barker 1999; Eberhard *et al.* 2001; Kumazawa *et al.* 1996; Lee *et al.* 2001; Mindell *et al.* 1998). In avian taxa with duplicated control regions, both copies have been found between *cytb* and 12S (Bensch & Harlid 2000; Eberhard *et al.* 2001), therefore this segment of the genome in *T. cauta* was examined thoroughly for evidence that a duplication may be present. Long-range PCR from *cytb* to 12S (described above) was repeated, and by running the product through a lower concentration agarose gel (0.8-1.0%) I determined the product length to be approximately 6000 bp, as opposed to approximately 3000 bp as previously reported by Abbott & Double (2003b [Chapter I]).

Two methods were undertaken to determine whether the two control regions, which were provisionally referred to as CR1 and CR2 and presumed to contain the F1 and F2 fragments respectively, were present within the 6000 bp fragment from *cytb* to 12S. First, a restriction digest map was constructed, for which expected restriction sites were predicted using previously obtained sequence data (described above) and knowledge of how sequence variation between F1 and F2 copies would affect digestion profiles. *Nsi*I (Roche Applied Science) and *Fsp*AI (MBI Fermentas) were used: the former cuts both putative copies of the control region twice, once within F1 and F2 and again at the end of domain II in both putative control regions, and the latter cuts the F1 copy once. Available sequence data suggested that these enzymes

did not have other cut sites within the 6000 bp fragment. As a second method of determining whether two control regions were located in this fragment, I attempted to PCR amplify the hypothetical region separating them using primer pairs PheF5/GluR7 and PheF6/GluR7 (Table 1, Figure 1). The locations and orientations of these primer pairs are such that products are expected only if two control regions are present (Figure 1). Both reactions used the same PCR reagent mix as described for the cbF1/12sR1 primer pair in Abbott & Double (2003b [Chapter I]) and followed these cycling parameters: 94°C for 2 min; 30 cycles of 15 s at 94°C, 30 s at 55°C, 2 min at 72°C and one final cycle for 7 min at 72°C.

To investigate the patterns of evolution in F1 and F2 sequences, DNA was extracted from each of *T. steadi*, *T. salvini*, and *T. eremita*, the three closest relatives of *T. cauta*, and *T. bulleri* (as outgroup) to determine whether they contained similar sequences. Indeed, unique cloned sequences (327 bp in length) corresponding to the F1 and F2 copy found in *T. cauta* were detected in all four species, as described in Abbott & Double (2003b [Chapter I]). These five sequences were used for phylogenetic analyses using unweighted cladistic parsimony, transition-weighted parsimony, and maximum likelihood using models optimized for the data in MODELTEST 3.06 (Posada & Crandall 1998). Tree estimates and subsequent assessment of their robustness by bootstrapping (Felsenstein 1985) were done using PAUP\*4.0b10 (Swofford 2002). As an additional means of evaluating their patterns of evolution, the uncorrected pairwise differences between F1 and F2 copies within individuals for each ingroup species were calculated using ARLEQUIN Version 2.001 (Schneider *et al.* 2000) and subsequently converted to sequence divergence estimates for whole and sectioned sequences.

## Results and Discussion

In this study I investigated the source of apparent sequence heteroplasmy in domain I of the mitochondrial control region of *Thalassarche cauta* to distinguish between three main hypotheses: co-amplification of a mitochondrial copy and a nuclear copy, the presence of a mtDNA duplication involving the control region, and the co-existence of non-identical mtDNA molecules within individuals (ie. mtDNA heteroplasmy).

F1 and F2 sequences were found to be maternally inherited in every mother/chick pair ( $n = 10$ ), implying a mitochondrial origin. The father was sampled in seven of these comparisons and in six of them his F1 and F2 sequence types differed from those of the mother (and chick). This analysis also revealed linkage between F1 and F2: any given F1 haplotype always accompanied the same F2 haplotype within individuals, which implied that they were on the same mtDNA molecule. The possibility of maternal inheritance mediated by W chromosome linkage was excluded as both male ( $n = 4$ ) and female ( $n = 6$ ) chicks inherited both maternal haplotypes.

Restriction digest profiles of the 6000 bp PCR product from *cytb* to 12S were consistent with the presence of two control regions separated by approximately 1100 bp. Subsequent PCR reactions targeting the region separating the two presumed control regions using primer pairs PheF5/GluR7 and PheF6/GluR7 each generated single products of lengths 1400 bp and 1700 bp, respectively, which were precisely concordant with expected sizes based on mapping results. Forward and reverse sequencing were carried out on both fragments, and results were combined with earlier sequencing results and restriction digest mapping to confirm the presence of a duplicated region which included the control region, several tRNA genes, the ND6 gene, and both degenerate and partial copies of the *cytb* gene (Figure 1). The gene



content differed from both the typical avian gene order (Figure 1) and from other gene rearrangements previously reported in birds (see Bensch & Harlid 2000; Eberhard *et al.* 2001). Sequence was obtained for most of this fragment, however sequence for 320 bp in the middle of ND6(2) (Figure 1) was not obtained due to insufficient read lengths of the forward and reverse reactions, and complete sequencing of domain III of CR2 (Figure 1) was precluded by long complex repeats (see below).

### Description of Duplicated Region

The alteration in mtDNA gene organisation reported here is comprised of tandemly duplicated sequence occurring between the first control region (CR1) and tRNA<sup>Phe</sup> (Figure 1). The duplicated section begins with d-cy**tb**, a 120 bp section that aligns with 70% sequence similarity to the complete cy**tb** sequence for *T. cauta* (Nunn *et al.* 1996) and is therefore considered a degenerate partial copy of this gene. d-cy**tb** is followed immediately by p-cy**tb** (Figure 1), which is a partial copy of the last 39 bp of cy**tb** that, unlike d-cy**tb**, is identical to the functional copy of this gene. Although they are immediately adjacent to one another in the duplicated region, in the functional cy**tb** gene the sequences corresponding to d-cy**tb** and p-cy**tb** are separated by 424 bp. Duplicate copies of tRNA<sup>Thr</sup>, tRNA<sup>Pro</sup>, and tRNA<sup>Glu</sup> (Figure 1) are identical to one another in all three cases. ND6(2) was not sequenced fully (see above), but its length is identical to ND6(1) and it is positioned between identical duplicates of tRNA genes. The lack of degeneration in duplicated tRNA genes (and potentially ND6) suggests that both copies of each gene may be functional, and contrasts with the results of other similar tRNA gene duplications (e.g. Arndt & Smith 1998; Campbell & Barker 1999; Eberhard *et al.* 2001; Kumazawa *et al.* 1998).

### Comparison of CR1 and CR2 and Evaluation of their Functionality

The two control regions found in *T. cauta* aligned easily and without indels but were not identical in length or in sequence. The total length of CR1 was 973 bp whereas CR2 was estimated to be up to 2300 bp, the latter being largely due to what appears to be an approximately 1300 bp section of repetitive sequence in domain III of CR2 (described below). Until recently, the largest reported avian control region was 1240 bp (greenfinch; Baker & Marshall 1997), but control regions ranging from 1868 bp to 2040 bp have since been reported in parrots (*Amazona* spp.; Eberhard *et al.* 2001) and penguins (Slack *et al.* 2003) respectively.

The control region is involved in the regulation of replication and transcription of the mitochondrial genome (Ruokonen & Kvist 2002) and its structural organisation is essentially the same across all organisms (Sbisa *et al.* 1997). The degree of similarity of each *T. cauta* control region copy with conserved sequence elements putatively thought to be of functional importance was evaluated for evidence of degeneration. The goose hairpin (Quinn & Wilson 1993) consisting of a string of mononucleotide cytosine repeats that is characteristic of the end of the control region adjacent to tRNA<sup>Glu</sup> was found in both CR1 and CR2. Conserved extended termination associated sequences (ETAS) are characteristic of domain I and are thought to be where the recently synthesized nascent heavy strand stops in mtDNA replication (Sbisa *et al.* 1997). Both *T. cauta* control regions were aligned with the consensus mammalian sequences for ETAS1 and ETAS 2 described in (Sbisa *et al.* 1997). The level of sequence similarity of CR1 with ETAS 1 and ETAS 2 was 67% and 68% respectively, and that of CR2 with ETAS 1 and ETAS 2 was 70% and 62% respectively. The central domain II region was invariable between CR1 and CR2, and exhibited 82%, 88%, and 86% sequence similarity with conserved chicken sequences F box, D box, and C box (Southern *et al.* 1988) respectively. Domain III begins with a conserved sequence block, CSB1, which is thought to either contain or be adjacent to the site of origin of heavy strand replication (Sbisa *et al.* 1997). Both

*T. cauta* control regions contain a CSB1 with 89% sequence similarity to the chicken CSB1. To summarise, all comparisons of CR1 and CR2 in *T. cauta* with chicken and mammalian conserved sequences generated equivalent results for both copies, and are comparable to those obtained in similar comparisons of ostensibly functional duplicate control regions in parrots (Eberhard *et al.* 2001).

Despite these similarities, CR1 and CR2 were not identical in length or sequence. The length of domain I (from the start of the goose hairpin to the start of F box) was 350 bp in both CR1 and CR2 and contained variable bases between them. Domain II (from the start of the F box to the start of CSB1) in CR1 and CR2 was 448 bp long and identical in sequence. Domain III (from the start of CSB1 to the end of the control region) differed more markedly between the two control regions. CR1 domain III was 175 bp in length, did not contain repeated sequences, and ended at *cytb* which was the start of the duplicated region. These 175 bp of domain III of CR1 were identical to the start of this domain in CR2 however the latter appeared to extend for approximately 1300 bp (until tRNA<sup>Phe</sup>) in the form of complex repeats. The repetitive nature of this sequence precluded complete sequencing, however partial sequence was obtained using primers PheF5 and 12sR2 (Table 1, Figure 1), and revealed two CA rich repeat motifs that were 23 bp and 44 bp in length. Similar CA rich repeat regions have been described in domain III of other albatrosses (Burg 2000) and avian taxa including penguins (Slack *et al.* 2003, Ritchie & Lambert 2000) and alcids (Moum *et al.* 2002).

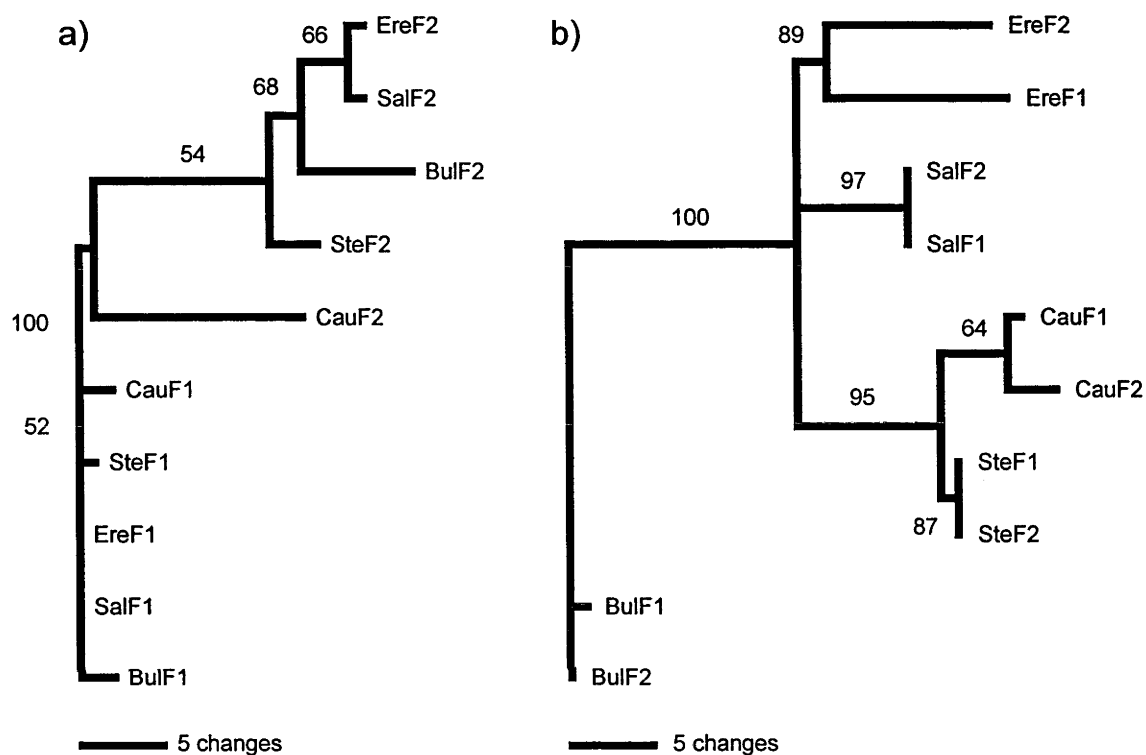
It is difficult to assess how the absence of domain III repeats in CR1 and the abundance of them in CR2 may impact on the functionality of either one. Tandemly repeated sequences are a common feature of the control region and are thought to be involved in the termination of transcription (Lunt *et al.* 1998). However, considering that they are not ubiquitous and that all other sequence comparisons between CR1

and CR2 did not uncover differences likely to affect their functionality, my results do not identify either copy as being degenerate.

### Phylogenetic Analyses of F1 and F2

Sequence variation between the duplicate control regions in *T. cauta* was only observed in the F1 and F2 fragments (Figure 1), hence these regions alone were used for phylogenetic analyses. As described by Abbott & Double (2003b [Chapter I]), a recombination signal was detected in all F1 copies using the reverse successive weighting method of Trueman (1998) and a larger sample size than used here. This resulted in conflicting phylogenetic signals from different portions of the sequences such that trees generated using all 327 bp for F1 and F2 in all five species were unresolved apart from separation of the outgroup (not shown) despite obvious variation among the sequences. Both the F1 and F2 copies for all individuals were therefore partitioned at the recombination site found in F1 which yielded 110 bp called section A and an adjacent 217 bp called section B.

Much higher resolution was achieved in trees produced using sections A and B separately (Figure 2), which remarkably depicted opposite evolutionary patterns between them. If the two copies were evolving independently since before speciation of the group, orthologous copies (ie. all F1s and all F2s) would be more closely related to one another than would paralogous copies (ie. F1 and F2 within individuals). The predicted phylogenetic tree under this scenario would have well-supported separation between an F1 clade and an F2 clade, which was the observed result for section A (Figure 2). In contrast, if the two copies were evolving in concert, paralogous copies would be more closely-related to one another than orthologous copies, leading to phylogenetic trees with well-supported terminal nodes connecting the two copies within individuals. This result was obtained for section B (Figure 2). Therefore my results suggest that one section of domain I of the



**Figure 2.** Unweighted cladistic parsimony phylogenetic trees for control region 1 (called F1) and control region 2 (called F2) of *Thalassarche cauta* (Cau), *T. steadi* (Ste), *T. salvini* (Sal), and *T. eremita* (Ere), with *T. bulleri* (Bul) as the outgroup. Sequences were partitioned into section A (110 bp; tree labelled as a) and section B (217 bp; tree labelled as b) as they generated different phylogenetic signals. Bootstrap values were obtained from 1000 resamplings.

duplicate control regions in these albatrosses is evolving independently, and the other section is evolving in concert. This pattern was also clearly portrayed by divergence estimates: the average uncorrected sequence divergence between F1 and F2 within individuals in the four ingroup taxa was 13.4% in section A and 0.6% in section B. An intriguing result that I have found difficult to explain was that branch lengths in Figure 2 clearly indicate that section A of F1 is evolving more slowly than the rest of F1 and all of F2.

### **Maintenance of CR1 and CR2**

Tandem duplication of mitochondrial DNA may arise via slipped-strand mispairing during DNA replication (Moritz & Brown 1986; Moritz & Brown 1987; Moritz *et al.* 1987). It was traditionally thought to be followed by the random loss of supernumerary genes (Moritz *et al.* 1987), whereby the coexistence of duplicated regions was short-lived on an evolutionary time scale (Moritz & Brown 1987). This has received some empirical support, with an apparently degenerate second copy of the control region found in representatives of four orders of birds (Mindell *et al.* 1998). However, recent studies on parrots (Eberhard *et al.* 2001), snakes (Kumazawa *et al.* 1996; Kumazawa *et al.* 1998), metastriate ticks (Black & Roehrdanz 1998; Campbell & Barker 1999), sea cucumbers (Arndt & Smith 1998), and self-fertilising fish (Lee *et al.* 2001) have described duplicate control regions that show no signs of degeneration but rather appear to be evolving in concert. Hence Kumazawa *et al.* (1996) have suggested that mtDNA molecules with duplicated control regions may be favoured by selection, perhaps due to a functional advantage arising from increased efficiency in the initiation of replication or transcription from multiple sites. This idea has also been endorsed by Campbell & Barker (1999) and Arndt & Smith (1998).

The mechanism responsible for the concerted evolution of duplicate control regions is unclear. The majority of studies identify frequent gene conversion as the most plausible possibility (e.g. Black & Roehrdanz 1998; Eberhard *et al.* 2001; Kumazawa *et al.* 1996). This process homogenises two sequences through nonreciprocal recombination whereby one sequence is completely replaced by another relevant sequence (Kumazawa *et al.* 1996). While gene conversion occurs frequently in nuclear genomes its occurrence in mitochondrial genomes is unproven (Kumazawa *et al.* 1996). A hypothetical description of how it may result in concertedly-evolving control regions is provided by Eberhard *et al.* (2001). An alternative mechanistic hypothesis is the tandem duplication model described by Kumazawa *et al.* (1998).

To my knowledge, results presented here on the mitochondrial gene order in *Thalassarche* albatrosses and the contrasting evolutionary patterns within their duplicate control regions have not been reported for any other taxa. While section A of CR1 and CR2 are evolving independently, the rest of the alignable portions of the two control regions are evolving in concert, and I suggest that the same may be true for the identically-duplicated tRNA and ND6 genes sequenced in *T. cauta*. I speculate that gene conversion is a plausible mechanism behind these evolutionary patterns. Multiple recombination points associated with a gene conversion mechanism could exist whereby certain duplicated portions are regularly homogenized such that they evolve in concert over the long term while intervening regions (e.g. section A) remain unaffected and therefore evolve independently.

### **Taxonomic Distribution of Duplication Event**

Deep phylogenetic origins are evident in other mitochondrial DNA duplications that have led to two concertedly evolving copies of the control region. Shared duplications of this kind have been described within: (1) snakes belonging to three families (Kumazawa *et al.* 1996); (2) several members of a sea cucumber family

(Cucumariidae; Arndt & Smith 1998); and (3) all four subfamilies of metastriate ticks (Black & Roehrdanz 1998).

The mitochondrial DNA duplication revealed here in five *Thalassarche* species may also be taxonomically widespread. Evolutionary studies of three other *Thalassarche* species (*T. melanophrys*, *T. impavida*, and *T. chrysostoma*; Burg & Croxall 2001) and three *Diomedea* species of albatrosses: *D. exulans*, *D. antipodensis*, and *D. dabbenena* (M.D. unpub. data; Burg & Croxall 2004) also found sequence heteroplasmy in domain I of the control region (T. Burg, pers. comm). The distribution of heteroplasmic sites in these species is very similar to that found in this study (data not shown) and are therefore likely to have a common cause. Indeed, anecdotal evidence suggests that the duplication may be present in all tube-nosed seabirds (i.e. order Procellariiformes): sequence heteroplasmy in domain I has been found in northern fulmars (*Fulmarus glacialis*; T. Burg, pers. comm.) and band-rumped storm-petrels (*Oceanodroma castro*; A. Smith, pers. comm.). A Procellariiform phylogeny presented by Kennedy & Page (2002) indicates that the genus *Oceanodroma* separated from a major clade containing *Fulmarus* and all albatross genera during the first divergence event in the common ancestor of the order. Therefore the mitochondrial duplication reported here is likely common to all Procellariiformes and could extend into neighbouring phylogenetic groups, which include penguins, loons, and frigatebirds (Sibley & Ahlquist 1990). Control region studies are scarce for these groups, although the standard gene order has been reported in two species of penguin (*Eudyptula minor*, Slack *et al.* 2003; *Pygoscelis adeliae*, Ritchie & Lambert 2000). Curiously however, low levels of single site heteroplasmy have been reported in domain I of the control region in razorbills (*Alca torda*; Moum & Bakke 2001). This could reflect a mitochondrial duplication, despite some phylogenetic distance from Procellariiformes (Sibley & Ahlquist 1990). In conclusion, I recommend that results of this study be considered in future evolutionary studies targeting the control region of all Procellariiformes and



potentially other closely-related avian groups, and encourage further research into identifying the full phylogenetic distribution of this mitochondrial duplication.

## References

- Abbott CL, Double MC (2003a) Genetic structure, conservation genetics, and evidence of speciation by range expansion in shy and white-capped albatrosses. *Molecular Ecology*, **12**, 2953-2962.
- Abbott CL, Double MC (2003b) Phylogeography of shy and white-capped albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy. *Molecular Ecology*, **12**, 2747-2758.
- Arndt A, Smith MJ (1998) Mitochondrial gene rearrangement in the sea cucumber genus *Cucumaria*. *Molecular Biology and Evolution*, **15**, 1009-1016.
- Baker AJ, Marshall HD (1997) Mitochondrial control region sequences as tools for understanding evolution. In: *Avian Molecular Evolution and Systematics* (ed. Mindell DP), pp. 51-82. Academic Press, New York.
- Bensch S, Harlid A (2000) Mitochondrial genomic rearrangements in songbirds. *Molecular Biology and Evolution*, **17**, 107-113.
- Black WC, Roehrdanz RL (1998) Mitochondrial gene order is not conserved in arthropods: prostriate and metastriate tick mitochondrial genomes. *Molecular Biology and Evolution*, **15**, 1772-1785.
- Burg T (2000) *Genetic Analyses of Albatrosses: Mating Systems, Population Structure and Taxonomy*. PhD thesis, Cambridge University, UK.
- Burg TM, Croxall JP (2001) Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology*, **10**, 2647-2660.
- Burg TM, Croxall JP (2004) Global population structure and taxonomy of the wandering albatross species complex. *Molecular Ecology*, **13**, 2345-2355.

- Campbell NJH, Barker SC (1999) The novel mitochondrial gene arrangement of the cattle tick, *Boophilus microplus*: fivefold tandem repetition of a coding region. *Molecular Biology and Evolution*, **16**, 732-740.
- Desjardins P, Morais R (1990) Sequence and gene organization of the chicken mitochondrial genome: a novel gene order in higher vertebrates. *Journal of Molecular Biology*, **212**, 599-634.
- Eberhard JR, Wright TF, Bermingham E (2001) Duplication and concerted evolution of the mitochondrial control region in the parrot genus *Amazona*. *Molecular Biology and Evolution*, **18**, 1330-1342.
- Felsenstein J (1985) Confidence limits on phylogenies: an approach using the bootstrap. *Evolution*, **39**, 783-791.
- Grzybowski T (2000) Extremely high levels of human mitochondrial DNA heteroplasmy in single hair roots. *Electrophoresis*, **21**, 548-553.
- Kennedy M, Page RDM (2002) Seabird supertrees: combining partial estimates of procellariiform phylogeny. *Auk*, **119**, 88-108.
- Kidd MG, Friesen VL (1998) Sequence variation in the guillemot (Alcidae: *Cepphus*) mitochondrial control region and its nuclear homolog. *Molecular Biology & Evolution*, **15**, 61-70.
- Kumazawa Y, Ota H, Nishida M, Ozawa T (1996) Gene rearrangements in snake mitochondrial genomes: highly concerted evolution of control-region-like sequences duplicated and inserted into a tRNA gene cluster. *Molecular Biology and Evolution*, **13**, 1242-1254.
- Kumazawa Y, Ota H, Nishida M, Ozawa T (1998) The complete nucleotide sequence of a snake (*Dinodon semicarinatus*) mitochondrial genome with two identical control regions. *Genetics*, **150**, 313-329.
- Lee JS, Miya M, Lee YS, *et al.* (2001) The complete DNA sequence of the mitochondrial genome of the selffertilizing fish *Rivulus marmoratus* (Cyprinodontiformes, Rivulidae) and the first description of duplication of a control region in fish. *Gene*, **280**, 1-7.

- Lunt DH, Whipple LE, Hyman BC (1998) Mitochondrial DNA variable number tandem repeats (VNTRs) - utility and problems in molecular ecology. *Molecular Ecology*, **7**, 1441-1455.
- Mindell DP, Sorenson MD, Dimcheff DE (1998) Multiple independent origins of mitochondrial gene order in birds. *Proceedings of the National Academy of Sciences of the United States of America*, **95**, 10693-10697.
- Moritz C, Brown MW (1986) Tandem duplication of D-loop and ribosomal RNA sequences in lizard mitochondrial DNA. *Science*, **233**, 1425-1427.
- Moritz C, Brown WM (1987) Tandem duplications in animal mitochondrial DNAs: variation in incidence and gene content among lizards. *Proceedings of the National Academy of Sciences of the United States of America*, **84**, 7183-7187.
- Moritz C, Dowling TE, Brown WM (1987) Evolution of animal mitochondrial DNA: relevance for population biology and systematics. *Annual Review of Ecology and Systematics*, **18**, 269-292.
- Moum T, Arnason U, Arnason E (2002) Mitochondrial DNA sequence evolution and phylogeny of the Atlantic Alcidae, including the extinct great auk (*Pinguinus impennis*). *Molecular Biology and Evolution*, **19**, 1434-1439.
- Moum T, Bakke I (2001) Mitochondrial control region structure and single site heteroplasmy in the razorbill (*Alca torda*; Aves). *Current Genetics*, **39**, 198-203.
- Nunn GB, Cooper J, Jouventin P, Robertson CJR, Robertson GG (1996) Evolutionary relationships among extant albatrosses (Procellariiformes, Diomedidae) established from complete cytochrome-*b* gene sequences. *Auk*, **113**, 784-801.
- Posada D, Crandall KA (1998) MODELTEST: testing the model of DNA substitution. *Bioinformatics*, **14**, 817-818.
- Quinn TW, Wilson AC (1993) Sequence evolution in and around the mitochondrial control region in birds. *Journal of Molecular Evolution*, **37**, 417-425.

- Ritchie PA, Lambert DM (2000) A repeat complex in the mitochondrial control region of Adelie penguins from Antarctica. *Genome*, **43**, 613-618.
- Ruokonen M, Kvist L (2002) Structure and evolution of the avian mitochondrial control region. *Molecular Phylogenetics and Evolution*, **23**, 422-432.
- Ruokonen M, Kvist L, Lumme J (2000) Close relatedness between mitochondrial DNA from seven *Anser* goose species. *Journal of Evolutionary Biology*, **13**, 532-540.
- Sbisa E, Tanzariello F, Reyes A, Pesole G, Saccone C (1997) Mammalian mitochondrial D-loop region structural analysis: identification of new conserved sequences and their functional and evolutionary implications. *Gene*, **205**, 125-140.
- Schneider S, Kueffer JM, Roessli D, Excoffier L (2000) *ARLEQUIN, Version 2.000. A Software Program for Population Genetic Data Analysis*. Genetics and Biometry Laboratory, University of Geneva, Geneva.
- Sibley CG, Ahlquist JE (1990) *Phylogeny and Classification of Birds: A Study in Molecular Evolution*. Yale University Press, New Haven, CT.
- Slack KE, Janke A, Penny D, Arnason U (2003) Two new avian mitochondrial genomes (penguin and goose) and a summary of bird and reptile mitogenomic features. *Gene*, **302**, 43-52.
- Sorenson MD, Fleischer RC (1996) Multiple independent transpositions of mitochondrial DNA control region sequences to the nucleus. *Proceedings of the National Academy of Sciences of the United States of America*, **93**, 15239-15243.
- Sorenson MD, Quinn TW (1998) Numts: a challenge for avian systematics and population biology. *Auk*, **115**, 214-221.
- Southern SO, Southern PJ, Dizon AE (1988) Molecular characterization of a cloned mitochondrial genome. *Journal of Molecular Evolution*, **28**, 32-42.
- Swofford DL (2002) *PAUP\*. Phylogenetic Analysis Using Parsimony (\*and Other Methods)*. Sinauer Associates, Sunderland, MA.

Trueman JWH (1998) Reverse successive weighting. *Systematic Biology*, **47**, 733-737.

Zhang DX, Hewitt GM (1996) Nuclear integrations: challenges for mitochondrial DNA markers. *Trends in Ecology & Evolution*, **11**, 247-251.



# Future Directions



Many new and interesting possibilities emerged from the research described in this thesis, some of which I am currently pursuing. Here I briefly highlight some profitable avenues for further research.

In Chapter III, I evaluated the relative degree of vulnerability of shy and white-capped albatrosses to fisheries bycatch mortality across all continental shelf regions thought to be regularly visited by these species. Towards the goal of gaining kill rate information for these species, I am now in the process of combining current fishing effort data for all major fisheries known to catch shy-type albatrosses with available information of their foraging distributions. The degree to which each species overlaps with each fishery will be predicted, and the associated risks of that exposure inferred. Nel *et al.* (2002) recently conducted this type of study on one population of wandering albatrosses (*Diomedea exulans*), and uncovered high spatial overlap between the birds and both tuna and Patagonian toothfish longline fisheries. Sex-specific patterns and seasonal variation in extent of overlap with these fisheries were found (Nel *et al.* 2002).

The full phylogenetic distribution of the mitochondrial duplication that I describe in five *Thalassarche* albatrosses requires further investigation. While researching for this study it was fascinating to discover just how many other reports of similarly 'strange' patterns of apparent sequence heteroplasmy had been found in the same region of other taxa. Information on the evolutionary origin and phylogenetic spread of the duplication, and whether it has been lost in any taxa, would be highly relevant to future studies using mtDNA to reconstruct phylogenies of procellariiform seabirds, and perhaps more distantly related birds.

For white-capped albatrosses, how the small (50-100 pairs; Gales 1998), and geographically distant, population of at Bollons Island (within the Antipodes Island group) fits into the genetic landscape of this species is unknown. It was not sampled



in this study due to the difficulties accessing the breeding birds. The island is difficult to land on, and reaching the birds would require abseiling 50 m down a cliff face to a small ledge where the nests are located. Yet if sampling this group were achieved, it would be possible to assess whether geographic distance is an isolating mechanism for white-capped albatrosses. The finding that shy albatrosses arose from white-capped albatrosses suggests that geographic distance may indeed isolate populations and promote speciation in these birds. However, the alternative possibility of genetic panmixia has been found among geographically distant populations of multiple albatross species (Burg & Croxall 2001, 2004).

The need for detailed studies on white-capped albatrosses existed before this thesis was born, however I hope my work has highlighted its urgency. Apart from work presented in this thesis, available published scientific papers on this species are limited to a comparison of their morphometry with shy albatrosses (Double *et al.* 2003), and an account of pig predation at their breeding site on Auckland Island (Flux 2002). Knowledge of temporal and spatial variation in the at-sea distribution of white-capped albatrosses is needed to identify seasons and/or geographic regions of particularly high risk of fisheries-related mortality, and data on the demography and status of their populations would assist in measuring its impact. Information on white-capped albatross breeding biology and confirmation of whether they are annual or biennial breeders is also critical to understanding the conservation implications of bycatch mortality for this species.

More broadly, much more could be learned from further genetic studies on other albatross species. In particular, genetic data are needed to clarify the recognition of two species of yellow-nosed albatrosses (*Thalassarche chlororhynchos* and *T. carteri*) and Buller's albatrosses (*T. bulleri* and *T. nov. sp.*). Second, despite the information gained from fine scale genetic studies on individual albatross species presented in this thesis and by Burg & Croxall (2001, 2004), we do not have a clear

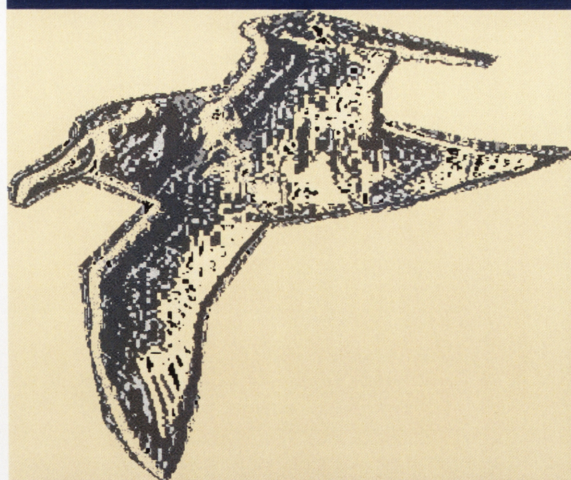


general understanding of albatross dispersal, migration, and speciation patterns. The question of why some taxa are limited to a single island (e.g. *Diomedea dabbenena* and *T. impavida*) and others are globally distributed (e.g. *D. exulans* and *T. chrysostoma*) persists. Perhaps interpreting information on the genetic structure of populations in the context of data on foraging patterns, courtship, and mating behaviour for several individual species would shed light on what factors are promoting divergence among some albatross populations and inter-migration among others. Ultimately this could resolve the number of species and speciation processes among extant albatrosses.

## References

- Burg TM, Croxall JP (2001) Global relationships amongst black-browed and grey-headed albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology*, **10**, 2647-2660.
- Burg TM, Croxall JP (2004) Global population structure and taxonomy of the wandering albatross species complex. *Molecular Ecology*, **13**, 2345-2355.
- Double MC, Gales R, Reid T, Brothers N, Abbott CL (2003) Morphometric comparison of Australian shy and New Zealand white-capped albatrosses. *Emu*, **103**, 287-294.
- Flux IA (2002) New Zealand white-capped mollymawk (*Diomedea cauta steadi*) chicks eaten by pigs (*Sus scrofa*). *Notornis*, **49**, 175-176.
- Gales R (1998) Albatross populations: status and threats. In: *Albatross Biology and Conservation* (eds. Robertson G, Gales R), pp. 20-45. Surrey Beatty & Sons, Chipping Norton.
- Nel DC, Ryan PG, Nel JL, *et al.* (2002) Foraging interactions between wandering albatrosses *Diomedea exulans* breeding on Marion Island and long-line fisheries in the southern Indian Ocean. *Ibis*, **144**, E141-E154.

# Appendix





## Morphometric comparison of Australian Shy and New Zealand White-capped Albatrosses

Michael C. Double<sup>A,E</sup>, Rosemary Gales<sup>B</sup>, Tim Reid<sup>B,C</sup>, Nigel Brothers<sup>B,D</sup> and Cathryn L. Abbott<sup>A</sup>

<sup>A</sup>Evolutionary Ecology Group, Division of Botany and Zoology, Australian National University, Canberra, ACT 0200, Australia.

<sup>B</sup>Marine Conservation Unit, Nature Conservation Branch, DPIWE, Box 44, Hobart, Tas. 7001, Australia.

<sup>C</sup>Current address: South-west Atlantic Seabirds at Sea Team, Falkland Conservation, PO Box 705, Stanley, Falkland Islands.

<sup>D</sup>Current address: Marine Ecology and Technology, PO Box 81, Kettering, Tas. 7155, Australia.

<sup>E</sup>To whom correspondence should be addressed. Email: Mike.Double@anu.edu.au

**Abstract.** Albatrosses are frequently killed by longline and trawl fishery operations but the relative impact of such activities at the species or population level are largely unknown. Such information requires the widespread presence of fishery observers and an ability to identify accurately the species and provenance of all albatrosses killed by fishing vessels. In this study we investigate the use of morphometric measurements to identify Shy (*Thalassarche cauta*) from White-capped (*T. steadi*) Albatrosses, two taxa recently suggested to be separate species. Measurements were taken from a collection of 103 Shy and White-capped Albatrosses killed by longline vessels within the Australian Fishing Zone between 1988 and 2000 and identified to species level using a recently developed DNA-based test. Within-sex comparisons of Shy and White-capped Albatrosses found that six of the 10 measurements were significantly different for both sexes. However, all measurements showed considerable overlap and no single measurement separated the two taxa. Discriminant classification functions based on wing chord, maximum head width and two bill measurements were able to simultaneously identify the species and sex of approximately 84% of bycatch specimens ( $n = 70$ ). The discriminate classification functions for species identification alone correctly assigned approximately 89% of bycatch specimens. When this classification method was applied to measurements taken from live specimens a similar level of accuracy was achieved (82%,  $n = 17$ ).

### Introduction

The decline of many albatross populations has been linked to the incidental killing of albatrosses by longline (Prince *et al.* 1994; Weimerskirch *et al.* 1997; Croxall *et al.* 1998; Gales 1998; Stehn *et al.* 2001; Woehler *et al.* 2001) and trawl (Bartle 1991; Weimerskirch *et al.* 2000) fishery operations. Such findings have led to pressure for fishing vessels to adopt bycatch mitigation measures (Brothers *et al.* 1999a, 1999b; Cooper 2000; Melvin and Parrish 2001) and for the widespread deployment of on-board observers to record fisheries-related mortality of seabirds (Brothers *et al.* 1999a; Baker *et al.* 2002; Ryan *et al.* 2002).

Widespread observer coverage would greatly improve bycatch estimates and also provide the opportunity to refine impact assessments at the species or population level. Such detail, however, requires accurate identification of both the species and provenance of all albatrosses killed by fishing vessels, a process that is confounded by our current lack of understanding of the species and population structure within the albatross family. Recently, an 'interim taxonomy' was suggested by Robertson and Nunn (1998) which elevated the

number of genera within the Diomedidae from two to four and the total number of species from 14 to 24. There appears to be general agreement with the generic-level revisions, but there has been greater debate about the newly designated species boundaries (James 2000; Warham 2001; Bourne 2002). This contention prompted fine-scale genetic studies which attempted to resolve the terminal taxa within the albatross family (Burg 2000; Burg and Croxall 2001; Abbott and Double 2003a). These studies have largely confirmed the existence of the terminal taxa identified by Robertson and Nunn (1998) but it remains arguable whether the genetic distances between some taxa warrant specific status (Burg 2000; Burg and Croxall 2001; Abbott and Double 2003a).

One of the most controversial suggestions made by Robertson and Nunn (1998) was to assign specific status to Shy (*Thalassarche cauta*) and White-capped (*T. steadi*) Albatrosses. Falla (1933) first proposed them as distinct subspecies but they were described as 'doubtfully separable' by Marchant and Higgins (1990), who lumped them together as a single subspecies within the *Diomedea cauta* complex. Robertson and Nunn (1998) referred to differences in the breeding

ology and non-overlapping wing morphometrics to the recognition of Shy and White-capped Albatrosses as separate species (hereby collectively referred to as 'shy-type'). Later genetic studies (Abbott and Double 2003a, b) confirmed that Shy and White-capped Albatrosses are closely related but did not detect any gene flow between the two taxa. This result highlights the need to treat these taxa as distinct units for assessments of anthropogenic mortality. Both Shy and White-capped Albatrosses are known to be targeted by longline fishing operations around Australia (Brothers 1991; Brothers and Foster 1997; Gales *et al.* 1998), New Zealand (Murray *et al.* 1993) and South Africa (Ryan 2002). Ryan *et al.* (2002) suggested that the shy-type albatrosses caught around South Africa were predominantly White-capped Albatrosses, according to the distinguishing measurement suggested by Robertson and Nunn (1998). The distinguishing wing measurement was, however, refuted by Holdaway *et al.* (2001). In this paper we attempt to redress this confusion by presenting morphological data taken from a collection of shy-type albatrosses caught in the Australian Fishing Zone (cf. Gales *et al.* 1998; Hedd *et al.* 1998). The carcasses were identified as either Shy or White-capped Albatrosses using a recently developed DNA-based test (Double *et al.* 2001; Abbott and Double 2003a, b). The principal aim of this study was to construct a reliable protocol to distinguish Shy from White-capped Albatrosses using morphometrics alone; the result also represents the first detailed morphometric analysis of the two taxa.

## Methods

### Species and nomenclature

The most recent taxonomic hypothesis available for albatrosses was adopted: the taxonomy suggested by Robertson and Nunn (1998). The names 'Shy Albatross' for *Thalassarche cauta* and 'White-capped Albatross' for *T. steadi* and, although under scrutiny, for ease of comparison refer to them here as separate species.

Shy Albatrosses breed exclusively on three islands around Australia. Approximately, 5000 pairs breed on Albatross Island, 7000 pairs on The Mewstone and 200 pairs on Pedra Branca (Gales 1998). Prior to breeding for the first time Shy Albatrosses are far from Australian waters (Brothers *et al.* 1997) but adults usually remain close to the breeding islands (Brothers *et al.* 1998; 1999). Most White-capped Albatrosses breed within the Indian Ocean Island group: Disappointment Island (70 000–80 000 pairs), Christmas Island (3000 pairs) and Adams Island (100 pairs) (Croxall *et al.* 1998; Taylor 2000). White-capped Albatrosses also breed on Christmas Island in the Antipodes Islands group (~100 pairs: Tennyson *et al.* 1998) and in the Chatham Islands on Forty-Four Island (one pair: Tennyson 2000). Little is known about the dispersal and foraging behaviour of White-capped Albatrosses, but molecular analyses have distinguished White-capped Albatrosses among birds killed by fisheries in the Indian, New Zealand and South African waters (C. Abbott and M. C. Double, unpublished data).

### Molecular identification of provenance

This study is based on shy-type albatrosses present in a collection of specimens collected between 1988 and 2000 by observers on board long-vessels operating within the Australian Fishing Zone (cf. Gales

*et al.* 1998; Hedd *et al.* 1998). At the time of this study the collection held 70 whole specimens, plus 19 where only the head and one lower limb had been retained, and 14 head-only specimens. All specimens had been frozen prior to measuring.

Samples of DNA extracted from each specimen were used to identify the bird as either a Shy or White-capped Albatross. The molecular identification technique is based on a consistent difference in the mitochondrial DNA of Shy and White-capped Albatrosses (Double *et al.* 2001) and is described in full by Abbott and Double (2003a). The general applicability of the molecular test was verified using blood samples taken from 10 albatrosses breeding on each of the three islands around Tasmania and 10 from each of the three islands in the Auckland Islands group. The test correctly identified the regional origin of all 60 samples (Abbott and Double 2003a).

The sex of bycatch specimens was identified by dissection or by a DNA-based sex test (Griffiths and Tiwari 1995; Norris-Caneda and Elliot 1998).

### Measurements and scores

The 10 body measurements presented here are largely from the same data set presented previously by Hedd *et al.* (1998). Differences are due to the addition of new data, remeasuring or the correction of errors introduced during data transcription. A detailed description of the measurement protocol and nomenclature is given by Hedd *et al.* (1998). Briefly, we took the following measurements using Vernier calipers ( $\pm 0.1$  mm): culmen length (CL), upper bill depth (UBD), minimum bill depth (MBD), basal bill depth (BBD), basal bill width (BW), maximum head width (MHW) and head length (HL). Tarsus length was measured with the foot bent over to the 'notch' in the intertarsal joint. The middle toe was measured on the upper side from the base of the claw to the joint between the toe and the tarsus. Wing chord (WC) was measured using a tape rule ( $\pm 1$  mm) from the carpal joint to the tip of the longest primary. We have changed 'wing length' of Hedd *et al.* (1998) to 'wing chord' to avoid possible confusion with full wing measurements. We have not presented measurements that cannot be accurately taken from live specimens (e.g. wing span) or that are likely to have low discriminatory power between closely related species (e.g. body mass). Following Hedd *et al.* (1998), we have pooled measurements taken from adult and subadult birds.

The colouration of the bill of each specimen was recorded using two separate scores. Shy-type albatrosses lose the black or dark grey colouration of the maxillary and mandibular unguis with age (N. Brothers, unpublished data; Hedd *et al.* 1998) although a few breeding adults have been recorded with a dark tip to their mandibular unguis (Hamilton *et al.* 2000). Each carcass was given a score ranging from 0 to 5 to reflect this beak colouration: 0 – no black/dark grey in bill; 1 – some dark colouration in mandibular unguis; 2 – strong dark colouration in mandibular unguis; 3 – strong dark colouration in mandibular unguis and some in maxillary unguis; 4 – strong dark colouration in both the mandibular and maxillary unguis; 5 – both the mandibular and maxillary unguis are black or dark grey. Specimens with bill scores greater than zero were classified as subadults (Hedd *et al.* 1998).

We also scored the amount of yellow colouration at the proximal end of the culmen. This feature is thought to differ between the two species with adult White-capped Albatrosses showing less yellow at the

**Table 1. Species, sex and age composition among the 103 shy-type specimens**

Age class	Shy Albatross		White-capped Albatross	
	Male	Female	Male	Female
Adults	20	30	4	9
Subadults	4	11	8	17

base of the culmen than Shy Albatrosses (Falla 1933; Marchant and Higgins 1990). The scoring regime was as follows: 0 – no yellow at the base of the culmen; 1 – some yellow; 2 – strong yellow colouration. Scores were allocated without prior knowledge of each specimen’s identity. This scoring was done opportunistically (by MCD) on 69 of the 103 specimens when the collection was transferred to a different storage facility in 2002.

The morphometric data from the shy-type bycatch specimens are available from RG.

Statistics

SPSS ver. 10 (SPSS Inc.) and JMP ver. 3.0.2 (SAS institute Inc.) statistical software were used to analyse the morphometric data. Probability values less than 0.05 were assumed to infer biological significance. We tested the normality of each measurement using the Shapiro–Wilk W test.

Discriminant function analysis (DFA) is a statistical classification method that uses data from known groups (e.g. sex or species) to construct a mathematical model that maximises the probability of correctly identifying the group origin of any new data. We conducted two separate DFAs: the first aimed to identify the species and sex simultaneously whereas the second aimed to identify species alone. For each analysis all measurements were initially entered into the model and those that did not contribute to the discriminatory power of the predictive model were sequentially removed.

The approximate discriminatory power of the species-and-sex DFA was tested by reclassifying the bycatch specimens. This was done in the same way that any new specimen would be classified. That is, by calculating a classification score for each of the four classification groups (e.g. male Shy Albatross, female Shy Albatross, etc.) using the

Table 2. Morphometric analysis of Shy and White-capped Albatrosses  
All data are in millimetres

Parameter	Shy Albatross		White-capped Albatross		Between-species ANOVA	
	Male	Female	Male	Female	Males	Females
Head length (HL)						
n	23	41	12	26		
Mean (s.d.)	83.9 (4.1)	80.8 (2.7)	87.0 (3.7)	84.4 (2.7)	F = 4.8	F = 28.2
Range	74.7–92.0	75.5–90.2	83.3–96.6	79.1–89.5	P < 0.05	P < 0.001
Maximum head width (MHW)						
n	24	41	12	26		
Mean (s.d.)	71.2 (1.5)	67.1 (1.3)	72.4 (1.0)	68.6 (1.5)	F = 7.0	F = 17.3
Range	68.5–74.8	64.1–69.8	70.4–74.0	65.0–71.9	P < 0.05	P < 0.001
Culmen length (CL)						
n	24	41	12	26		
Mean (s.d.)	136.1 (3.5)	132.3 (3.1)	137.5 (1.3)	133.5 (4.0)	F = 1.0	F = 1.7
Range	129.7–143.3	125.8–140.7	130.9–146.5	124.5–139.9	P = 0.3	P = 0.2
Basal bill width (BW)						
n	24	41	12	26		
Mean (s.d.)	35.0 (0.9)	33.0 (0.9)	34.7 (1.7)	33.0 (0.8)	F = 0.4	F = 0.08
Range	33.3–36.9	31.3–35.2	31.7–36.6	32.0–34.4	P = 0.5	P = 0.8
Basal bill depth (BBD)						
n	24	41	12	26		
Mean (s.d.)	54.8 (1.7)	51.6 (1.2)	54.9 (2.4)	52.6 (1.4)	F = 0.01	F = 9.4
Range	51.9–59.0	48.8–54.5	51.6–59.3	49.0–55.0	P = 0.9	P < 0.01
Minimum bill depth (MBD)						
n	24	41	12	26		
Mean (s.d.)	31.1 (1.2)	29.2 (0.8)	30.3 (1.1)	28.9 (0.9)	F = 3.4	F = 1.6
Range	28.9–33.4	27.3–31.2	28.9–32.6	27.1–30.5	P = 0.07	P = 0.2
Upper bill depth (UBD)						
n	24	41	12	26		
Mean (s.d.)	34.2 (1.0)	32.2 (0.7)	33.3 (1.3)	31.8 (1.1)	F = 5.4	F = 4.0
Range	32.5–36.1	30.4–34.4	31.2–35.3	29.0–32.9	P < 0.05	P < 0.05
Tarsus length						
n	19	33	9	23		
Mean (s.d.)	89.9 (3.1)	87.4 (2.0)	92.5 (1.7)	89.9 (2.7)	F = 6.0	F = 15.8
Range	84.0–95.6	82.6–87.4	88.8–94.8	85.4–94.9	P < 0.05	P < 0.001
Mid-toe length						
n	20	35	11	23		
Mean (s.d.)	119.7 (3.0)	116.6 (2.9)	124.4 (3.0)	119.0 (3.5)	F = 17.4	F = 8.2
Range	112.8–126.6	111.0–121.0	120.0–130.0	113.2–127.4	P < 0.001	P < 0.01
Wing chord (WC)						
n	14	26	10	20		
Mean (s.d.)	570.8 (16.1)	562.9 (11.5)	602.3 (12.6)	580.6 (14.6)	F = 26.5	F = 21.0
Range	540.0–595.0	540.0–583.0	586.0–625.0	550.0–610.0	P < 0.001	P < 0.001

original morphometric data and classification coefficients derived from the DFA (cf. Tables 2 and 4):

$$\text{Classification score} = \text{constant} + c1(\text{MHW}) + c2(\text{WC}) + c3(\text{BBD}) + c4(\text{UBD}),$$

where  $c1$ ,  $c2$  ... are the classification coefficients for each body measurement retained in the DFA (cf. Table 4). Each specimen was assigned to the group that gave the highest classification score.

The classification coefficients derived from the species-only DFA (cf. Table 6) were tested using measurements taken from 17 shy-type albatrosses that were caught alive at sea off Wollongong, New South Wales, by the Southern Ocean Seabird Study Association (SOSSA). These birds were identified to species using the molecular test described above.

## Results

The molecular test revealed that the bycatch collection contained 65 Shy and 38 White-capped Albatrosses. Table 1 shows the species, sex and age-class composition of the 103 specimens.

### *Morphological characteristics of bycatch specimens*

Normality tests of the 10 morphological measurements grouped by sex and species indicated that three of 40 groups

did not conform to the expectations of a normal distribution. However, because each of the three significant results fell into different measurement categories (UBD, HW and HL) that displayed normality in the three other species/sex categories we decided not to transform the original data.

Within-sex comparisons of Shy and White-capped Albatrosses showed significant differences for both sexes in six of the 10 morphological measures (Table 2). In addition, measurements of the BBD were significantly larger among female White-capped than among female Shy Albatrosses. Generally, Shy Albatrosses were smaller than White-capped Albatrosses; only the UBD measurement was found to be significantly smaller among White-capped Albatrosses (Table 2, Fig. 1). Despite the significant statistical comparisons, all measurements showed considerable overlap (Fig. 1), including those subsequently employed by the DFA analyses described below.

No White-capped Albatrosses were recorded with any yellow colouration at the base of the culmen even though the sample contained nine adults that had little or no dark colouration in the unguis (Fig. 2). In contrast, the scoring of the Shy Albatross specimens indicates that the yellow colouration in

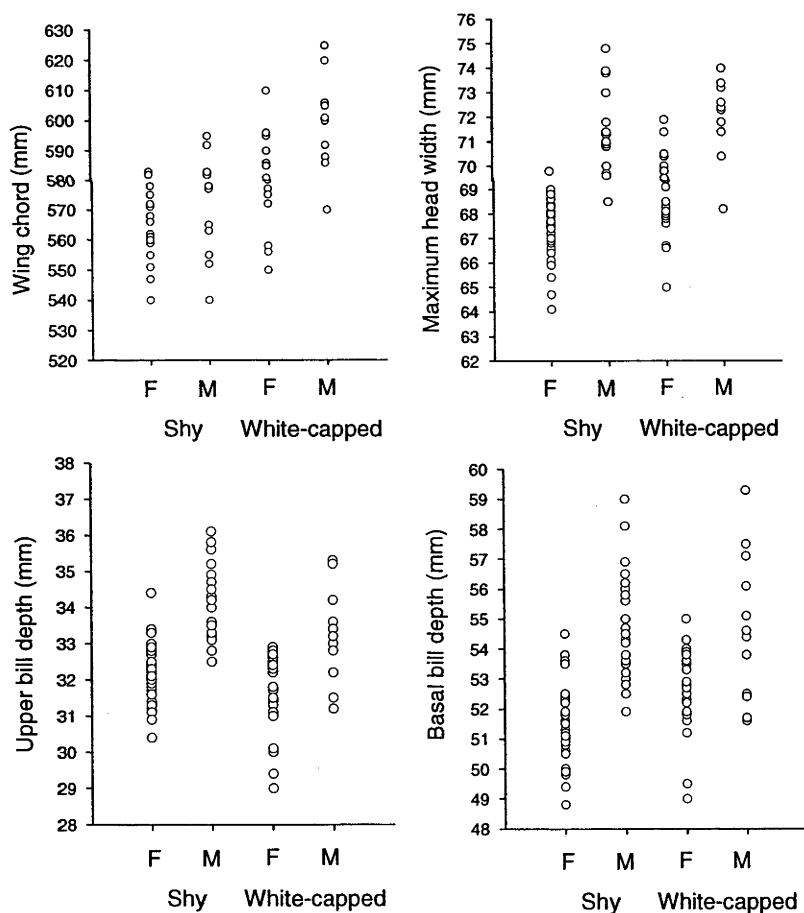


Fig. 1. Distribution of data from the four measurements taken from Shy and White-capped Albatrosses and used to discriminate species and sex. F = female; M = male.

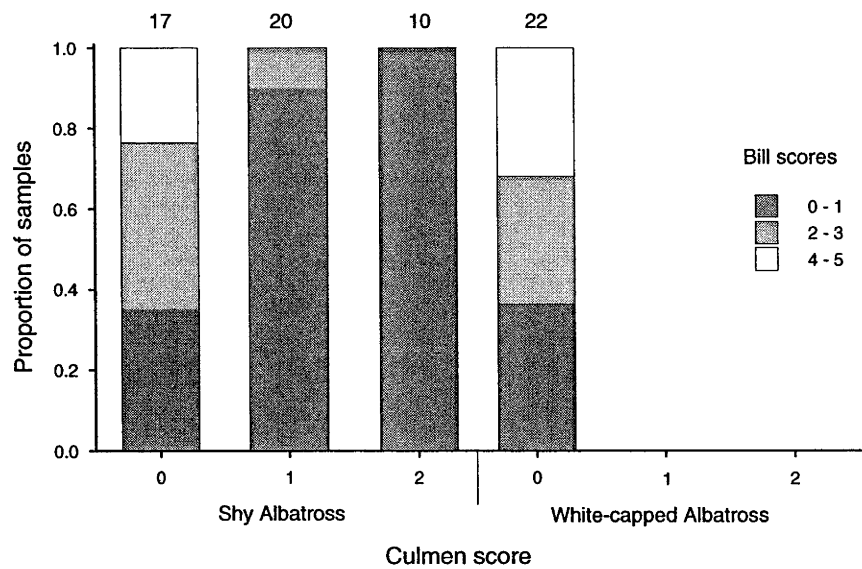


Fig. 2. Scoring of colouration in the bills of bycatch specimens. A culmen score of zero indicates no yellow colouration. Bill scores reflect the amount of black or dark grey in the unguis. A score of zero indicates an adult specimen. Sample sizes are given above each column. See Methods for full descriptions of the scoring protocols.

the culmen strengthens as the dark colouration of the unguis is lost. The highest annual proportion of Shy Albatross specimens with culmen scores of two had been frozen for six (30%,  $n = 18$ ) and eight years (20%,  $n = 11$ ). Thus there is no indication that the frozen specimens had lost colour with time.

Discriminant function analyses

Four measurements (MHW, UBD, BBD and wing chord; Fig. 1) remained in the discriminant function analysis to classify species and sex. The relative contributions of each

measurement to the three discriminant functions are presented in Table 3. MHW contributed most to the first function whereas UBD and BBD contributed most to the second and third functions respectively. The classification function coefficients (Table 4) correctly identified both the sex and species of 84.3% (59 of 70) of all specimens (Table 5). Species identity was correctly assigned to 61 of the 70 specimens (87.1% correct). Among the nine samples that were assigned to the wrong species group, four were Shy Albatrosses (three females and one male) and five were White-capped Albatrosses (all females). There was a tendency for subadults to be incorrectly assigned more frequently: 4 of (9.8%) adults compared with 5 of 29 (17.2%) subadults. Sex was identified correctly for all but 3 of the 70 specimens (95.7% correct).

We conducted a second DFA to distinguish species or sex. Three measurements (UBD, BBD and wing chord) remained in the single discriminant function. UBD had the highest standardised canonical discriminant function coefficient (−1.198) followed by wing chord (0.865) and BBD (8.2). The classification function coefficients (Table 6) correctly

Table 3. Standardised canonical discriminant function coefficients for species and sex identification

Morphological measure	Discriminant function		
	1	2	3
Maximum head width	0.772	0.227	−0.660
Wing chord	0.441	−0.636	0.541
Bill base depth	0.359	−0.367	−1.029
Upper bill depth	−0.222	0.995	0.834

Table 4. Classification function coefficients for species and sex identification

Morphological measure	Shy Albatross		White-capped Albatross	
	Female	Male	Female	Male
Maximum head width	23.775	25.465	24.454	26.086
Wing chord	2.616	2.628	2.718	2.808
Bill base depth	7.765	8.288	8.652	8.779
Upper bill depth	6.059	6.447	4.072	4.526
Constant	−1830.318	−1994.596	−1917.335	−2107.199

**Table 5.** Classification results for identification of sex (*n* = 70)  
82.9% of cases were classified correctly

Actual group membership	Predicted group membership			
	Shy Albatross		White-capped Albatross	
	Female	Male	Female	Male
Shy Albatross, female	23	0	3	0
Shy Albatross, male	1	12	1	0
White-capped Albatross, female	5	0	14	1
White-capped Albatross, male	0	0	0	10

identified 88.6% (62 of 70) of all specimens (Table 7). These classification function coefficients were subsequently applied to measurements taken from 17 live-caught shy-type albatrosses (SOSSA, unpublished data). The molecular test revealed this sample to contain 4 Shy Albatrosses and 13 White-capped Albatrosses, of which 14 (82.4%) were correctly identified using morphometrics alone. No MHW measurements were available for these birds so we could not apply the classification function coefficients from the first DFA.

**Discussion**

Shy and White-capped Albatrosses are morphologically very similar. Although most measurements showed a significant difference between the two species, all measurements overlapped considerably and no single characteristic separated the two taxa. However, discriminant classification functions based on wing chord, maximum head width and two bill measurements were able to identify successfully the species and sex of approximately 84% of all bycatch specimens. A DFA to classify species alone correctly identified approximately 89% of all bycatch specimens. When the same classification method was applied to measurements taken from live specimens a similar level of accuracy (82%) was achieved. This suggests that the measurement data presented here are generally representative of Shy and White-capped Albatrosses and that the classification methodology is robust even if the measurements are taken from live or dead specimens and by different observers.

The discriminant classification functions identified sex more successfully than species (96% correct). This finding concurs with the analysis presented by Hedd *et al.* (1998) that identified MHW and UBD as the most useful measurements to identify sex in shy-type albatrosses. The discriminant function coefficients described by Hedd *et al.* (1998)

were only slightly less accurate (93%) when applied to the data set presented here than the discriminant classification functions described above.

Our data suggest that even as adults White-capped Albatrosses do not develop any yellow colouration at the base of the culmen whereas the culmen of Shy Albatrosses develop a strong yellow colouration as the bird matures. However, because some adult Shy Albatrosses showed no yellow at the base of the culmen this character cannot be used to identify all adult birds. Also, the character is of little use for bycatch identification given the high proportion of immature shy-type birds reported among longline seabird bycatch in Australian and southern African waters (Gales *et al.* 1998; Ryan *et al.* 2002).

Waugh *et al.* (1999) used a DFA approach in a study of black-browed (*Thalassarche* spp.) and Grey-headed Albatrosses (*Thalassarche chrysostoma*) from four subantarctic islands. In these species they found overlapping but statistically significant morphological differences between islands for each of the three measures taken (culmen, tarsus and wing chord). Their sample of black-browed albatrosses included both the nominate Black-browed Albatross (*T. melanophrys*) and the recently recognised Campbell Albatross (*T. impavida*; previously known as *Diomedea melanophrys impavida*) so perhaps their result is not surprising. Interestingly, however, the Grey-headed Albatross also showed significant differences among islands even though recent genetic studies suggest no substructure within this species (Burg and Croxall 2001). For both Black-browed and Grey-headed Albatrosses the DFA largely failed to identify the island of origin of each sample. Only the provenance of Campbell Albatrosses was identified with any reliability (76% correctly identified, *n* = 86). The results of Waugh *et al.* (1999) and those presented here suggest that morphological

**Table 6.** Classification functions for species identification

Morphological measure	Classification coefficients		Discriminant function coefficients
	Shy Albatross	White-capped Albatross	
Bill base depth	8.044	8.885	0.396
Upper bill depth	1.733	-0.239	-0.929
Wing chord	2.346	2.471	0.59
Constant	-904.396	-956.681	-24.447



**Table 7.** Classification results for species identification (*n* = 70)  
88.6% of cases were classified correctly

Actual group membership	Predicted group membership	
	Shy Albatross	White-capped Albatross
Shy Albatross	37	5
White-capped Albatross	3	25

measurements may be useful for bycatch identification only to the species (or subspecies) level and other methods are required to identify provenance.

Waugh *et al.* (1999) suggest that morphological differences between populations reflect differences in chick-provisioning rates and foraging distances. They found that populations with a generally smaller body size had larger foraging distances and hence longer chick-rearing periods. The morphometry of Shy and White-capped Albatrosses is not consistent with this trend. Shy Albatrosses are smaller than White-capped Albatrosses yet as adults they forage very close to their breeding islands (Hedd *et al.* 2001). Alternatively, the size differences could be influenced by the wind conditions in the preferred foraging zones. Recently, Shaffer *et al.* (2001) suggested that the sexual dimorphism of Wandering Albatrosses (*Diomedea exulans*) may reflect the direction chosen by each sex when leaving their breeding islands. The flight performance of the smaller and lighter female is greatest in the lighter winds of subtropical areas whereas the larger, heavier males are more suited to the windier conditions of the antarctic and subantarctic. Hence, the size differences between Shy and White-capped Albatrosses may be driven by the latitudinal differences between the breeding islands, with adult Shy Albatrosses adopting a relatively sedentary, wind-independent foraging strategy (Brothers *et al.* 1998; Hedd *et al.* 2001).

Here we have shown that measurements taken from shy-type bycatch specimens can be used to identify both species and sex with a reasonable level of accuracy. Genetic analysis of bycatch specimens can potentially provide a greater level of accuracy (Edwards *et al.* 2001) but requires specialised skills and infrastructure which are not always available to those assessing bycatch rates. In combination, both morphological and genetic studies could provide reliable bycatch data for species and populations impacted by fishery operations, but only with reasonable observer coverage for all fishing fleets. Such information, coupled with long-term population monitoring, would allow us to direct efforts more effectively to mitigate fisheries-related mortality of albatrosses and other seabirds.

**Acknowledgments**

We thank Jodie Adamczewski, Barry Baker, Harry Battam, Sheryl Hamilton, Kathryn Medlock, Peter Milburn, David Pemberton, Gary Nunn, Chris Robertson, Debbie Robertson,

Lindsay Smith, AFMA and its fisheries observer program, CSIRO Wildlife Collection, Environment Australia, SOSSA and the Tasmanian Museum for their invaluable assistance, comments and suggestions.

**References**

Abbott, C. L., and Double, M. C. (2003a). Phylogeography of Shy and White-capped Albatrosses inferred from mitochondrial DNA sequences: implications for population history and taxonomy. *Molecular Ecology* 12, 2747–2758.

Abbott, C., and Double, M. C. (2003b). Genetic structure, conservation genetics, and evidence of speciation by range expansion in Shy and White-capped Albatrosses. *Molecular Ecology* 12, 2953–2962.

Baker, G. B., Gales, R., Hamilton, S., and Wilkinson, V. (2002). Albatrosses and petrels in Australia: a review of their conservation and management. *Emu* 102, 71–97.

Bartle, J. A. (1991). Incidental capture of seabirds in the New Zealand subantarctic squid fishery, 1990. *Bird Conservation International* 1, 351–359.

Bourne, W. R. P. (2002). The classification of albatrosses. *Australasian Seabird Bulletin* 38, 10–12.

Brothers, N. (1991). Albatross mortality and associated bait loss in the Japanese longline fishery in the Southern Ocean. *Biological Conservation* 55, 255–268.

Brothers, N. P., and Foster, A. B. (1997). Seabird catch rates: an assessment of causes and solutions in Australia's domestic tuna longline fishery. *Marine Ornithology* 25, 37–42.

Brothers, N. P., Reid, T. A., and Gales, R. P. (1997). At-sea distribution of Shy Albatrosses *Diomedea cauta cauta* derived from records of band recoveries and colour-marked birds. *Emu* 97, 231–239.

Brothers, N., Gales, R., Hedd, A., and Robertson, G. (1998). Foraging movements of the Shy Albatross *Diomedea cauta* breeding in Australia – implications for interactions with longline fisheries. *Ibis* 140, 446–457.

Brothers, N., Gales, R., and Reid, T. (1999a). The influence of environmental variables and mitigation measures on seabird catch rates in the Japanese tuna longline fishery within the Australian Fishing Zone, 1991–1995. *Biological Conservation* 88, 85–101.

Brothers, N. P., Cooper, J., and Løkkeborg, S. (1999b). The incidental catch of seabirds by longline fisheries: worldwide review and technical guidelines for mitigation. FAO Fisheries Circular No. 937, Rome.

Burg, T. (2000). Genetic analyses of albatrosses: mating systems, population structure and taxonomy. Ph.D. Thesis, University of Cambridge.

Burg, T. M., and Croxall, J. P. (2001). Global relationships amongst Black-browed and Grey-headed Albatrosses: analysis of population structure using mitochondrial DNA and microsatellites. *Molecular Ecology* 10, 2647–2660.

Cooper, J. (Ed.) (2000). Albatross and petrel mortality from longline fishing. International Workshop, Honolulu, Hawaii, Report and presented papers. *Marine Ornithology* 28, 153–190.

Croxall, J. P., and Gales, R. (1998). An assessment of the conservation status of albatrosses. In 'Albatross Biology and Conservation'. (Eds G. Robertson and R. Gales.) pp. 46–65. (Surrey Beatty: Sydney.)

Croxall, J. P., Prince, P. A., Rothery, P., and Wood, A. G. (1998). Population changes in albatrosses at South Georgia. In 'Albatross Biology and Conservation'. (Eds G. Robertson and R. Gales.) pp. 69–83. (Surrey Beatty: Sydney.)

Double, M. C., Lashko, A., and Abbott, C. (2001). Genetic profiling of Shy Albatross (*Thalassarche cauta*) populations. Report for Environment Australia. Australian National University, Canberra.

- Edwards, S. V., Silva, M. C., Burg, T., Friesen, V. L., and Warheit, K. I. (2001). Molecular genetic markers in the analysis of seabird bycatch populations. In 'Seabird Bycatch: Trends, Roadblocks, and Solutions'. (Eds E. F. Melvin and J. K. Parrish.) pp. 115–140. (University of Alaska Sea Grant: Fairbanks.)
- Falla, R. A. (1933). Notes on New Zealand petrels: with descriptions of new forms and some new records. *Records of the Auckland Institute and Museum* 1, 173–180.
- Gales, R. (1998). Albatross populations: status and threats. In 'Albatross Biology and Conservation'. (Eds G. Robertson and R. Gales.) pp. 20–45. (Surrey Beatty: Sydney.)
- Gales, R., Brothers, N., and Reid, T. (1998). Seabird mortality in the Japanese tuna longline fishery around Australia, 1988–1995. *Biological Conservation* 86, 37–56.
- Griffiths, R., and Tiwari, B. (1995). Sex of the last wild Spix's Macaw. *Nature* 375, 454.
- Hamilton, S., Gales, R., and Brothers, N. (2000). Shy Albatrosses in Australia: population and conservation assessment. Report for Environment Australia. DPIWE, Tasmania.
- Hedd, A. (1999). Foraging ecology of Shy Albatrosses *Thalassarche cauta* breeding in Australia: implications for interactions with longline fisheries. Ph.D. Thesis, University of Tasmania, Hobart.
- Hedd, A., Gales, R., and Brothers, N. (1998). Reliability of morphometric measures for determining the sex of adult and fledgling Shy Albatrosses, *Diomedea cauta cauta*, in Australia. *Wildlife Research* 25, 69–79.
- Hedd, A., Gales, R., and Brothers, N. (2001). Foraging strategies of Shy Albatross *Thalassarche cauta* breeding at Albatross Island, Tasmania, Australia. *Marine Ecology Progress Series* 224, 267–282.
- Holdaway, R. N., Worthy, T. H., and Tennyson, A. J. D. (2001). A working list of breeding bird species of the New Zealand region at first human contact. *New Zealand Journal of Zoology* 28, 119–187.
- James, D. J. (2000). Comments on albatross taxonomy and species concepts. *Australasian Seabird Bulletin* 36, 13–16.
- Marchant, S., and Higgins, P. J. (Eds.) (1990). 'Handbook of Australia, New Zealand and Antarctic Birds.' (Oxford University Press: Melbourne.)
- Melvin, E. F., and Parrish, J. K. (2001). 'Seabird Bycatch: Trends, Roadblocks and Solutions.' (University of Alaska Sea Grant: Anchorage.)
- Murray, T. E., Bartle, J. A., Kalish, S. R., and Taylor, P. R. (1993). Incidental capture of seabirds by Japanese Southern Bluefin Tuna longline vessels in New Zealand waters, 1988–1992. *Bird Conservation International* 3, 181–210.
- Norris-Caneda, K. H., and Elliot, J. D. (1998). Sex identification in raptors using PCR. *Journal of Raptor Research* 32, 278–280.
- Prince, P. A., Rothery, P., Croxall, J. P., and Wood, A. G. (1994). Population dynamics of Black-browed and Grey-headed Albatrosses *Diomedea melanophrys* and *D. chrysostoma* at Bird Island, South Georgia. *Ibis* 136, 50–71.
- Robertson, C. J., and Nunn, G. B. (1998). Towards a new taxonomy for albatrosses. In 'Albatross Biology and Conservation'. (Eds G. Robertson and R. Gales.) pp. 13–19. (Surrey Beatty: Sydney.)
- Ryan, P. G., Keith, D. G., and Kroese, M. (2002). Seabird bycatch by longline fisheries off southern Africa, 1998–2000. *South African Journal of Marine Science* 24, 103–110.
- Shaffer, S. A., Weimerskirch, H., and Costa, D. P. (2001). Functional significance of sexual dimorphism in Wandering Albatrosses, *Diomedea exulans*. *Functional Ecology* 15, 203–210.
- Stehn, R. A., Rivera, K. S., Fitzgerald, S., and Wohl, K. D. (2001). Incidental catch of seabirds by longline fisheries in Alaska. In 'Seabird Bycatch: Trends, Roadblocks, and Solutions'. (Eds E. F. Melvin and J. K. Parrish.) pp. 61–77. (University of Alaska Sea Grant: Fairbanks.)
- Taylor, G. A. (2000). Action Plan for Seabird Conservation in New Zealand. Part A: Threatened Seabirds. Threatened Species Occasional Publication No. 16, Biodiversity Recovery Unit, Department of Conservation, Wellington, New Zealand.
- Tennyson, A., Imber, M., and Taylor, R. (1998). Numbers of Black-browed Mollymawks (*Diomedea m. melanophrys*) and White-capped Mollymawks (*D. cauta steadi*) at the Antipodes Islands in 1994–95 and their population trends in the New Zealand region. *Notornis* 45, 157–166.
- Warham, J. (2001). A further comment on albatross taxonomy. *Australasian Seabird Bulletin* 37, 13–14.
- Waugh, S. M., Prince, P. A., and Weimerskirch, H. (1999). Geographical variation in morphometry of Black-browed and Grey-headed Albatrosses from four sites. *Polar Biology* 22, 189–194.
- Weimerskirch, H., Brothers, N., and Jouventin, P. (1997). Population dynamics of Wandering Albatross *Diomedea exulans* and Amsterdam Albatross *D. amsterdamensis* in the Indian Ocean and their relationships with long-line fisheries – conservation implications. *Biological Conservation* 79, 257–270.
- Weimerskirch, H., Capdeville, D., and Duhamel, G. (2000). Factors affecting the number and mortality of seabirds attending trawlers and long-liners in the Kerguelen area. *Polar Biology* 23, 236–249.
- Woehler, E. J., Cooper, J., Croxall, J. P., Fraser, W. R., Kooyman, G. L., Miller, G. D., Nel, D. C., Patterson, D. L., Peter, H.-U., Ribic, C. A., Salwicka, K., Trivelpiece, W. Z., and Weimerskirch, H. (2001). A statistical assessment of the status and trends of antarctic and subantarctic seabirds. Scientific Committee on Antarctic Research, Cambridge.

Manuscript received 11 April 2003; accepted 19 August 2003